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The Water Taste in Mammals.

By

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A few years ago one of us (ZOTTERMAN 1949) observed that water applied to the frog's tongue produces a massive volley of large fibre impulses in the glossopharyngeal nerve, and later studies proved that the discharge could be distinguished from those of the acid receptors or the salt receptors (ANDERSSON and ZOTTERMAN 1950). It seemed possible that a similar situation might exist in mammals. Certain support for such an assumption was obtained in the course of experiments performed by one of us (G. L.) in continuation of earlier studies on chemical transmission in taste fibre endings (LANDGREN, LILJESTRAND and ZOTTERMAN 1954). In these experiments it became necessary to determine the threshold value of taste for certain substances in man. The method of correct and false cases was applied as described by LEMBERGER (1908). The subject was required to compare a solution in tap water of the substance to be tested with tap water and decide which of the two samples contained the substance. For this purpose 5 ml of each were taken into the mouth and kept there a few seconds. After each test the mouth was washed with water of the same temperature as that of the samples. In each series 10 comparisons were made and the percentage of correct answers was evaluated, an uncertain answer being reckoned as 1/2 correct and 1/2 false. Since random answers will give on an average 50 per cent correctness, 75 per cent was chosen as indicating the threshold.

It soon turned out in conformity with results of earlier authors that the subject was able to differentiate between water and a salt solution at a concentration where no specific salt taste was obtained. Thus for sodium chloride 0.009 M gave a definite and 0.0035 M a very uncertain perception of salt, but 0.0026 M still elicited 75 per cent correct answers. The same was true for 0.0014 M sodium sulphate which had no salt taste. The bitter taste of calcium chloride disappeared at a concentration of a little above 0.0055 M, but it was possible to differentiate between 0.003 M and tap water. On the other hand 0.0035 M sodium chloride could not be distinguished from 0.003 M calcium chloride, nor from 0.002 M sodium sulphate. According to HÖBER and KIESOW (1898) as well as HAHN, KUCKULIES and TAEGER (1938) the salt taste of sodium chloride and sodium sulphate is caused by the anions, whereas in the case of calcium chloride it seems mainly to be due to the cations. The impossibility of differentiating between the dilute solutions mentioned indicates that some factor other than the specific action of different ions must come into play. This is illustrated by the fact that tap water (with a dry residue of 0.014 per cent) could be distinguished from distilled water (dry residue 0.0004 per cent) with a certainty of 100 per cent. This also held true after the tap water had been boiled for 5 minutes and then cooled.

In the old literature the flat taste of water was held to represent a special "sapor insipidus" or "sapor aquosus" (cp. ÖHRWALL 1891). According to HENLE (1880) it is characteristic of solutions containing less salt than the saliva. He maintains that the flat taste is for the sense of taste what black is for the sense of vision. This view was endorsed by ÖHRWALL, who defined it as "the perception that a perception is lacking when one would expect it to occur". An alternative interpretation was suggested, however, by the experiments on water taste in the frog quoted above. We therefore decided to study this question by recording the impulses from the taste fibres of the chorda tympani nerve in some mammals.

Technique and Procedure.

All together 12 cats, one dog and one pig were used. The animals were anaesthetized by intramuscular or intravenous injection of a chloralose-urethane solution (0.05 g chloralose and 0.25 g urethane in 7 ml Ringer's solution per kg body-weight).

In order to avoid the admixture of spikes from the numerous mechanoceptive fibres of the lingual nerve the action potentials were led off from the central part of the chorda tympani nerve which contains very few mechanoceptive fibres (ZOTTERMAN 1935). The mandible was resected as has been previously described and the leads were placed either on the intact chorda tympani or on fine strands from this nerve (ANDERSSON, LANDGREN, OLSSON and ZOTTERMAN 1950).

In order to avoid the stimulation of the endings of the relatively numerous warm fibres which run into the chorda tympani nerve (see DODT and ZOTTERMAN 1952) the test solutions were usually kept at a constant temperature as close as possible to that of the exposed tongue (26°—28° C). The solutions were applied by pouring a constant amount of 15 ml from a glass funnel upon the tongue in about 3 seconds. Between the tests the tongue was irrigated with Ringer's solution (26°—28° C) when not otherwise remarked.

Thus the solutions were all applied exactly in the same way and at the same temperature. Differences in the nervous response must therefore be due entirely to the chemical composition of the solutions and not to any thermal or mechanical stimulation.

Results.

As a rule a slight spontaneous electrical activity of the chorda tympani nerve, varying in intensity from one cat to another, was observed. If the tongue was washed with Ringer's solution of about body temperature (35°—37° C), a moderate increase of the action potentials for half a second or less was usually found (Fig. 1 A). This was presumably the result of a stimulation of thermoreceptors, since the effect disappeared more or less completely, if the Ringer solution was held at the indifferent temperature of 26°—28° C. A very slight effect might, however, still persist, indicating some stimulation of the chemoceptor mechanism. However, had the tongue been washed with distilled water shortly before the application of Ringer, there occurred a sudden volley of impulses which disappeared within about 1/5 of a second. If the Ringer test was repeated, this response did not reappear. These results are illustrated in Fig. 3 for the dog and in Fig. 4 for the cat. Sometimes, on the other hand, the application of Ringer's solution caused a further reduction of the spontaneous activity. Characteristic of the effect of Ringer's solution was that it always was very slight and of short duration.

The effect of distilled water was in sharp contrast to that of Ringer's solution. If it was applied at 35°—37° C, the same imme-

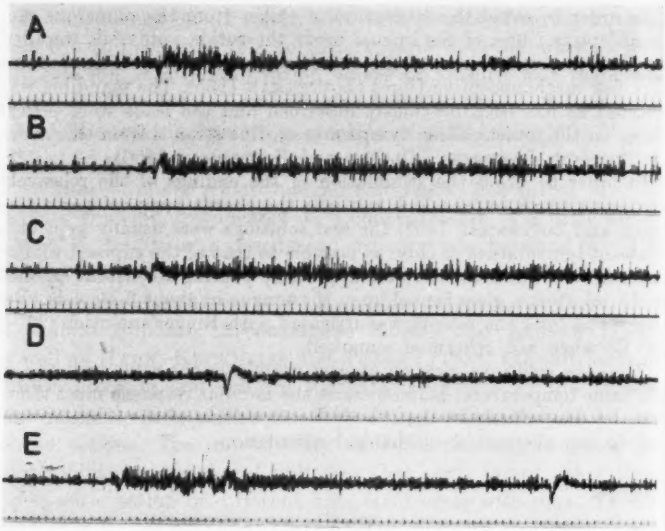

Fig. 1. All records in this paper depict the impulse activity from the unsplit chorda tympani nerve or some strands of this nerve. Time markings 50 c. per sec. in Fig. 1, 2, 4 and 5, 25 c. per sec. in Fig. 3. Cat, strands of the nerve. Temperature of solutions 35°—36° C. A Ringer's solution, B distilled water, C 0.3 M sucrose in water, D 0.3 M sucrose in Ringer's solution, E crystallose in Ringer's solution.

diate increase in the potentials as after Ringer occurred, but now it had a much longer duration (Fig. 1 B). If water was used at a temperature of 26°—28° C the action was about as strong as at the higher temperature and lasted during several seconds after the washing had been finished. On the whole the effect of distilled water was rather similar to that of a 0.5 M solution of sodium chloride. It had sometimes nearly the same intensity and duration as this solution (cf. Fig. 2 for the cat and Fig. 3 for the dog), though in other cases the salt solution mentioned acted definitely stronger. If, on the other hand, the sodium chloride concentration was diminished to 0.33 M, a considerably smaller effect resulted (Fig. 2 F), and with 0.17 M hardly any definite stimulation at all occurred (Fig. 2 G). Below that point the effect increased again. Thus, if Ringer's solution was diluted with distilled water, a gradual increase of the stimulating action resulted, as demonstrated in Fig. 2 B and C. Tap water had nearly as strong an effect

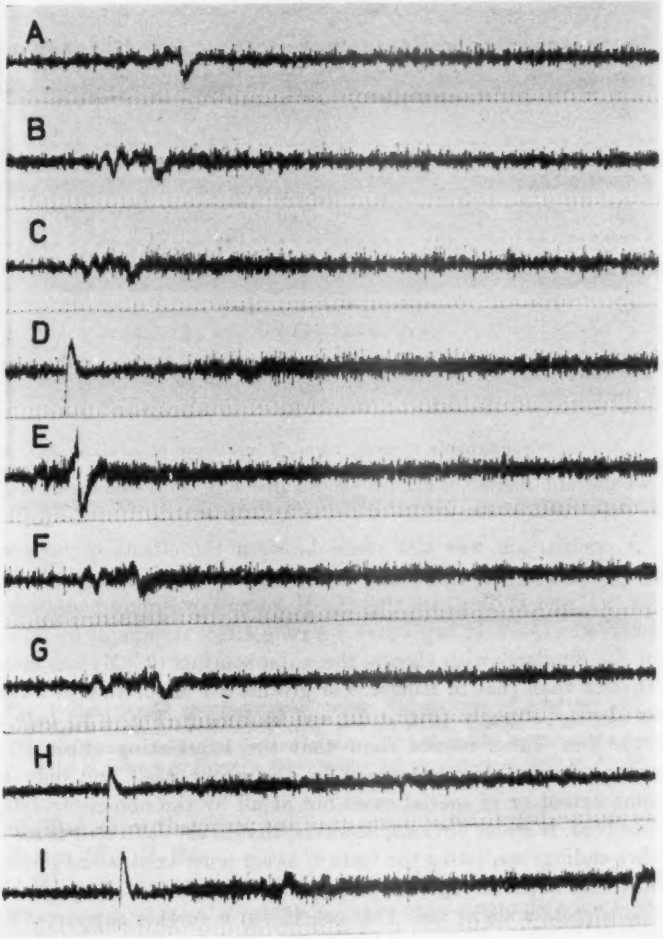

Fig. 2. Cat, unsplit chorda tympani nerve. Temperature of solutions 26.5° C. A Ringer's solution, B Ringer's solution diluted with 2 volumes of water, C Ringer's solution diluted with 5 volumes of water, D distilled water, E 0.5 M sodium chloride, F 0.33 M sodium chloride, G 0.17 M sodium chloride, H cotton pad soaked with distilled water, I cotton pad soaked with 0.5 M sodium chloride.

as distilled water. We have quite regularly observed the stimulating effect of water on the taste buds in more than 60 trials on our 12 cats and also in the dog and the pig.

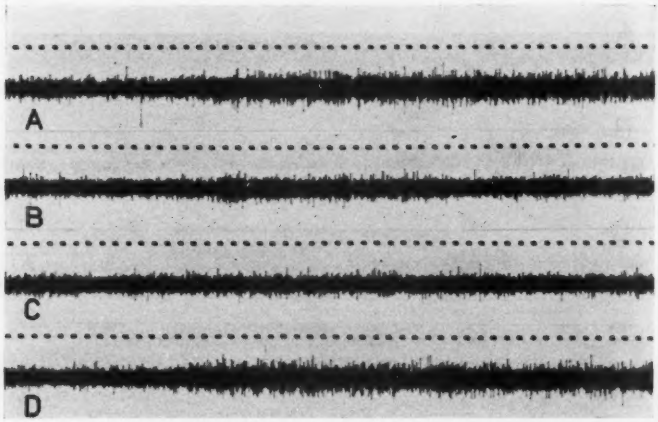

Fig. 3. Dog, unsplit chorda tympani nerve. Temperature of solutions 26.4° C. A distilled water, B Ringer's solution immediately after distilled water (without washing with Ringer!), C Ringer's solution after B, D 0.5 M sodium chloride.

A comparison was also made between the effects of sucrose, glucose and glycine, dissolved in water and in Ringer respectively. Fig. 1 C and D illustrate that 0.3 M sucrose in Ringer had a very slight effect, but in tap water a strong effect (temperature 35°—36° C). Similarly with glucose the water solution (0.3 M) was more efficient than that in Ringer. For glycine a 1 M solution in water acted very strongly (Fig. 4 D) and in Ringer (Fig. 4 E) only a little less. These results show that the stimulating effect of a water solution may be caused by the water itself and only to some extent or in special cases not at all by the non-electrolytes dissolved. It seems obvious, however, that in the cat's tongue some fibre endings mediating the taste of sweet must exist, since glycine in Ringer had a strong stimulating effect and glucose and probably also sucrose a slight one. This conclusion is further supported by the observation that a 0.6 M solution of sucrose in Ringer had a fairly strong stimulating action (Fig. 4 F). The same was observed with crystallose in Ringer (Fig. 1 E).

The results mentioned above have all been obtained with washing of the tongue. We also tried a less adequate technique: the application on the tongue of a cotton pad soaked with the different solutions. With this method a 0.5 M solution of sodium chloride gave nearly as strong a stimulation as washing (Fig. 2 I), but with

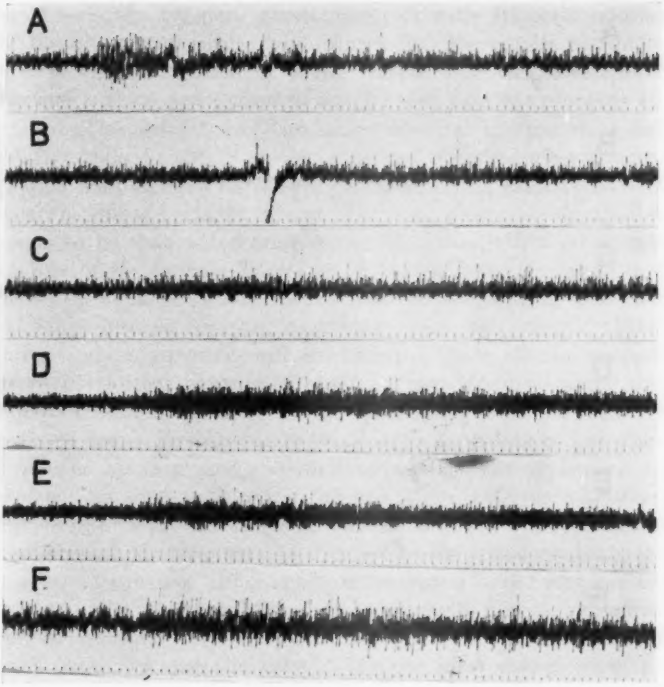

Fig. 4. Cat, unsplit chorda tympani nerve. Temperature of solutions 28.3° C. A Ringer's solution immediately after distilled water, B Ringer's solution after A, C distilled water, D 1 M glycine in water, E 1 M glycine in Ringer's solution, F 0.6 M sucrose in Ringer's solution (greater amplification than in A—E).

distilled water the effect was very much reduced under these conditions (Fig. 2 H).

For a further analysis of the water taste the chorda tympani nerve was split into fine strands, each containing only a few fibres, and the experiments were repeated.

As will be seen from Fig. 5, it was possible to obtain preparations where only water but not 0.5 M sodium chloride acted as a stimulating agent. In this case several different kinds of spikes appeared after the application of water, among them relatively large spikes which were not found after salt. Fig. 5 D and E furnish an example of the reversed situation: here salt induced an increased activity but water had no effect. In this case 1 M

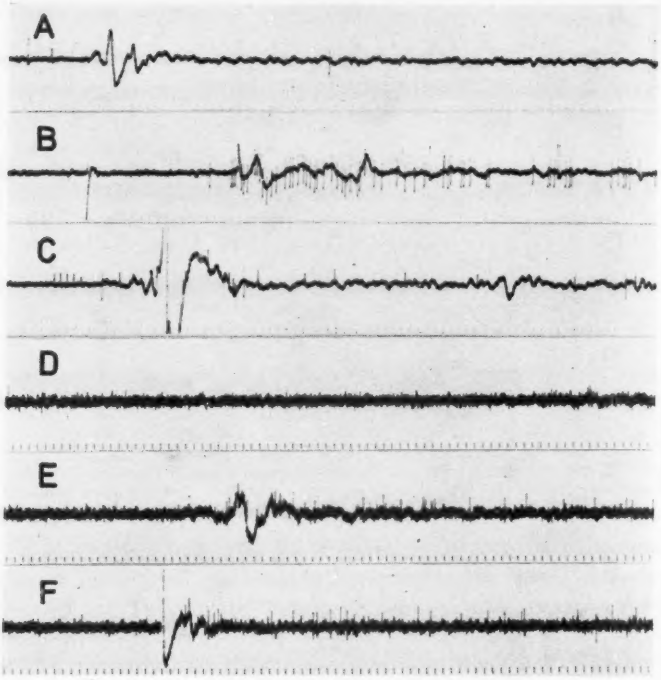

Fig. 5. A, B and C: Cat, few fibre preparation, temperature of solutions 27.8° C. D, E and F: another cat, few fibre preparation. Temperature of solutions 29.5° C. A Ringer's solution, B distilled water, C 0.5 M sodium chloride, D distilled water, E 0.5 M sodium chloride, F 1 M glycine in water.

glycine in water also proved stimulating. It must thus be assumed that the response to water is mediated by nerve fibres which are different from those responding to salt or to glycine. A similar conclusion was reached by ANDERSSON and ZOTTERMAN for the frog's tongue.

If a reaction was still evoked either by water or by salt, it was always possible to obtain a stimulating effect by the application of acid.

Discussion.

Washing the tip of the tongue of the cat, the dog or the pig with distilled water has been found to induce action potentials

in the chorda tympani nerve. Controls with Ringer's solution of the same temperature have shown that the results cannot be due to the stimulation of touch receptors or thermoreceptors. Neither is there any reason to assume that pain receptors are involved. The character of the spikes (general configuration and relative spike height) is the same as that observed after application on the tongue of the well-known chemical stimulants, sodium chloride, dilute acids, or glycine. The only interpretation possible seems to be that water exercises a stimulating effect on chemoceptors of the tongue. Therefore, a "water taste" similar to that described in frogs by ZOTTERMAN must be assumed. It is of considerable interest in this connection that PFAFFMANN (1941) in his study of gustatory afferent impulses in the chorda tympani nerve of the cat sometimes observed that distilled water was capable of calling out a transient response that could not be differentiated from the response to weak acid. According to him "the difference between acid and distilled water when applied to an acid ending — — — is not an 'all or nothing' difference between discharge and no discharge but rather a distinction between a discharge of one frequency as opposed to the transient discharge of a lower frequency". In our opinion the reason for the very modest response to water in Pfaffmann's experiments can be explained by the technique adopted by him. He applied the solutions with a small brush, so that the amount brought into contact with the tongue must have been rather small. Approximate ionic or osmotic equilibrium between a drop of water, the fluid on the surface and the superficial tissues of the tongue requires the diffusion of fewer ions or water molecules than the corresponding process with a drop containing 1 M sodium chloride as used in his experiments. In agreement herewith we observed a slight and transient effect only when a cotton pad soaked with water was applied to the tongue, whereas a strong response was elicited with 0.5 M sodium chloride. If, however, the applications were made with our method, where the surface of the tongue was continuously irrigated with the solutions, in both cases strong and very similar discharges followed.

In the white rat PFAFFMANN and BARE (1950), recording the action potentials from the chorda tympani nerve, found that "nearly all preparations gave some positive response to water". They maintain that very dilute solutions of salt failed to evoke a response (above that of water), whereas an increase of the concen-

tration from 0.05 to 1 per cent (and further) produced greater activity. From their experiments they give the mean threshold for sodium chloride as 0.0081 per cent. These results do not seem to agree very well with ours as we found that Ringer's solution or a solution of about 0.9 per cent of sodium chloride provoked a very slight activity whereas greatly enhanced responses from the whole nerve were obtained if the concentration was raised sufficiently from that level. The threshold values for sodium chloride as determined by PFAFFMANN and BARE in the white rat seem to us remarkably low even compared to the threshold values found in man. In this connection the results of RICHTER and MACLEAN (1939) should be remembered. They found in man an average sodium chloride threshold of 0.19 per cent with the drop method but 0.08 per cent when the subject had to compare water and salt solutions by taking 10 ml of each into his mouth. Furthermore, they give the threshold value for sodium chloride for normal rats as 0.055 per cent (based on experiments with choice between water and salt solutions). It is, however, very difficult to compare threshold values of the peripheral receptors to those determined by psycho-physical methods because in the latter case it is the central threshold which is measured. It may be that the interpretation of the results of PFAFFMANN and BARE should be revised in the light of our present observations. A similar revision must be considered for the results of BEIDLER (1953). These investigations, as well as others, have not taken into account the water response, although their own data might well have led them to this point of view.

By reducing the number of fibres in the chorda tympani nerve we have been able to obtain preparations which showed good response to distilled water but only an insignificant activity or none at all from 0.5 M sodium chloride. We have also obtained the opposite result, *i. e.* a fairly strong reaction from salt — and also from glycine — but none from water. In some instances spikes of a special type appeared after water but not after sodium chloride. These observations show that there must be different fibres responding to sodium chloride and to water. On the other hand we have regularly obtained positive results from acetic acid, when water or salt induced activity. This is in agreement with PFAFFMANN's finding that fibres responding to salt always reacted to acid as well. These responses could not be distinguished from each other, whereas in the frog the impulses after acid could be differ-

entiated from those after water or salt, since in the former case they had a much smaller spike height (ANDERSSON and ZOTTERMAN 1950).

According to our observations 0.15 M or 0.3 M solutions of sucrose in Ringer's fluid had at most only a very feeble stimulating effect on the taste receptors in the cat, whereas the same concentration of sucrose in water elicited strong responses. Similarly 0.3 M glucose in Ringer barely stimulated, but the corresponding solution in water induced great fibre activity. The conclusion must be drawn that the water taste cannot to any great extent be due to the osmotic differences between water and the receptors. It seems necessary to assume that it is mainly due to an exchange of ions. The stimulating action of water would then be similar to that of certain salts. If one assumes that this latter effect is due to the penetration of anions into the receptor cells (cp. TIMM 1950), one might suppose a wandering of the same ions in the opposite direction when water is applied. A stimulation would then occur when the resting ionic concentration in the receptors is altered to a certain extent in either direction. This would also explain the feeble and very transient stimulating effect of Ringer's solution, when it is applied shortly after irrigation with water but without previous washing with saline. In this case a temporary ionic gradient between the Ringer solution and the taste cells would have been created.

Since water induces a perception quite different from that of salts, it will be necessary to assume different nerve fibres in the two cases, one being stimulated by excess and the other by a lowering of the resting ionic concentration. It will be simplest to suppose that in certain cells the receptor mechanism has been so modified as to answer to an increase, in other cells on the contrary to a decrease in the concentration of the active ions. The other possibility, that a single taste cell should have a double innervation, one for each of the two conditions mentioned, seems less probable. The fact that all chemoceptors of the taste buds of the cat seem to be sensitive for acids might be interpreted in different ways. Thus the hydrogen ions might not only stimulate specific receptors but also by breaking through the membrane barrier of the non-specific receptors release a chain reaction leading to a general stimulation also of these other receptors, possibly also of pain receptors.

Our experiments have led to the conclusion that in the cat,

the dog, the pig and most likely in all mammals there are specific nerve endings responding to water which thus may constitute a mechanism for discrimination of water containing only very small amounts of electrolytes or none. The taste of water obviously cannot be explained as being exclusively analogous to darkness, resulting from the absence of an expected perception but must also be due to a direct stimulation of specific nerve endings, just as is the case with substances that induce acid, salt, bitter or — at least in some animals — sweet tastes.

Neglecting the stimulating rôle of water on taste receptors may easily lead to false conclusions with regard to the effect of dissolved substances. This is illustrated by our experiments with sucrose or glucose referred to above. The great increase of the electrical activity after application of water solutions of these sugars might erroneously be interpreted as due to stimulation of receptors for the taste of sweet. But since the effect nearly disappeared when Ringer's solution was used as the solvent, such an interpretation is unwarranted. There remained at least in some experiments a small effect even with glucose or sucrose in Ringer's solution, indicating that receptors for sweet also exist in the tongue of the cat. An increase of the concentration of sucrose to 0.6 M gave a somewhat more pronounced response. Furthermore, glycine in 1 M solution in Ringer had a fairly strong effect, nearly as great as in water, and crystallose in Ringer also led to increased activity. The smaller molecules of glycine and crystallose thus seem to be relatively more efficient in this case. On the whole, the results speak in favour of the view that in the cat there are receptors for sweet though probably not very numerous. The existence of such receptors is in agreement with the observations of FRINGS (1951) who found in cats some preference for diluted milk containing 0.5 M sucrose to milk without sucrose.

Summary.

The impulse activity of the chorda tympani nerve in the cat, the dog and the pig was only very slightly influenced by washing the tongue with Ringer's solution at about 26°—28° C. If the tongue had been irrigated with distilled water immediately before the Ringer test, a sudden volley of impulses occurred for about 1/5 of a second.

Washing the tongue with distilled water led to a considerable increase in the response, sometimes of about the same intensity as after a 0.5 M solution of sodium chloride.

It was possible to obtain few-fibre preparations from the chorda tympani which displayed increased impulse activity after application on the tongue of water but not of salt and also such strands as reacted to salt or glycine but not to water.

It is concluded that there are specific nerve endings — most likely in all mammals — responding to water containing no electrolytes or only very small amounts. The possible mechanism of the stimulation is discussed.

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Action of Estradiol Monobenzoate on the Mammary Glands of Hypophysectomized Rabbits.

By

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Received 16 June 1954.

It is well established that mammary gland growth is promoted by ovarian hormones in intact animals. In spite of extensive investigations in many species under different experimental conditions it is still difficult to assess the part played by the anterior pituitary gland in the processes evoked by ovarian hormones and resulting in growth and development of mammary glands (FOLLEY and MALPRESS 1948, FOLLEY 1952). From experiments on parabiotic rats JACOBSOHN, 1948, concluded "... that the ovarian hormones exert their action on the mammary gland directly as well as indirectly via the anterior pituitary". From work of others and their own FOLLEY and MALPRESS (1948) and FOLLEY (1952) deduce two possible mechanisms: 1) estrogens sensitize the mammary gland and release a stimulating factor from the anterior pituitary gland, and 2) estrogens release a sensitizing factor from the anterior pituitary gland and stimulate the mammary gland directly. The attempted analysis of an interplay between ovarian and anterior hypophysial hormones reflects the fact that the available evidence is in favour of the view that mammary gland growth cannot be stimulated by ovarian hormones alone.

However, in hypophysectomized animals injected with estrogens and progesterone mammary gland growth has been reported

to occur. For reasons summarized by FOLLEY and MALPRESS (1948) most of these older reports may be disregarded. It does, however, not seem justified to apply this attitude towards the work done on hypophysectomized rabbits.

ASDELL and SEIDENSTEIN (1935) studying 4 hypophysectomized castrated rabbits found "almost" the same mammary gland growth as in castrates after injections of theelin and progesterone. In 2 immature hypophysectomized rabbits GOMEZ and TURNER (1937) did not find any development of the mammary glands. FREDRIKSON (1939) examined 15 immature ovariectomized hypophysectomized rabbits after injections of estradiol monobenzoate, either alone or together with progesterone. In these animals FREDRIKSON obtained "... development of the structures of the mammary gland corresponding to that found in animals which have not been hypophysectomized ..." To the best of my knowledge further studies of that kind on rabbits have not been published.

The results obtained by the three investigators are not uniform. The communications of 1935 and 1939 would indicate that in the rabbit the mode of action of ovarian hormones on the mammary gland is different from that of other rodents.

After transection of the hypophysial stalk and injections of estradiol monobenzoate JACOBSOHN (1949) observed in rabbits mammary gland changes that were strikingly different from those of unoperated controls. The glands were widely distended with milk-like secretion. Proliferative processes, although difficult to estimate because of the distension, seemed to be fairly limited. The observations on stalk transected rabbits cannot easily be reconciled with those made by ASDELL and SEIDENSTEIN (1935) and FREDRIKSON (1939) on hypophysectomized rabbits.

In the present work the reaction of the mammary glands of hypophysectomized rabbits injected with estradiol monobenzoate is reinvestigated. Care was taken to avoid the fallacies pointed out during the last decade by investigators in this field.

Experimental.

The material presented consists of 71 mammary glands taken at different times during experiments on 14 completely and 17 incompletely hypophysectomized castrated juvenile and adult male and female rabbits injected with estradiol monobenzoate. Control experiments on castrated rabbits with the hypophysis left untouched are not given. Observations on animals showing a loss in body weight during the course of the experiments are not described either.

The rabbits were kept under uniform conditions, fed ad libitum with corn and greens in summer and with corn, hay and beets or carrots during winter. They were weighed at weekly intervals.

The hypophysectomy was made as described by JACOBSOHN and WESTMAN (1940). At autopsy the contents of the sella turcica together with the median eminence of the tuber cinereum of the brain were dissected out under magnifying glasses, embedded in paraffin wax and cut into serial sections of about 10 μ. The sections were stained with hematoxylin-eosin and examined microscopically. Remnants of the pituitary gland consisting only of zona tuberalis in contact with the brain tissue above the roof of the sella turcica are also regarded as indicating an incomplete hypophysectomy. In the completely hypophysectomized animals the pars tuberalis of the median eminence is still present.

The animals were castrated before the start of injections, either before or after hypophysectomy. Details are given below.

A preparation of estradiol monobenzoate in olive oil (Di Menformon[1]) was injected intramuscularly, the dose being 100 μg in 0.1 ml every 2nd day during the first fortnight and later every 4th day. In a few cases the injections were made at 4 day intervals from the beginning onwards.

Mammary glands were extirpated at different times during the experiments. At operation the presence and quality of secretion was recorded. The growth and development was studied on whole mount preparations stained with gallocyanin-chromalum and estimated as in previous work (JACOBSOHN 1948, 1949). In a few experiments thin, hematoxylin-eosin stained sections of parts of a mammary gland were examined microscopically. The experimental glands were compared with the corresponding glands removed before treatment from the other side of the same rabbit.

Results.

The experiments on females are summarized in tables 1 and 2, and figures 1—10 illustrate typical observations in both sexes.

Table 1: 14 mammary glands taken from 9 completely hypophysectomized females have been examined at 14 to 65 days after the first injection. The body weights had been kept fairly constant, a slight loss occurring in only 3 instances. All but 3 slightly distended glands were widely distended with a colourless secretion, a drop of which could easily be squeezed out through the nipple. On account of the distensions the degree of parenchymal growth was difficult to score with any accuracy. As may be seen from figures 1 to 3 (exp. 5 and 12), showing typical glands before and

[1] Kindly supplied by the Pharmacia Ltd., Uppsala.

Table 1.

Completely hyp.ect. female rabbits castrated 3 to 4 weeks before hyp.ect. Start of injections of estradiol monobenzoate 2 to 6 days after hyp.ect.

Exp.	Body weight at		Mammary glands examined after first injection		
	hyp.ect.	exam. of m. gl.			
	g	g	days	growth[1]	secretion
1	2,570	2,740	14	(+)	+
2	3,150	3,070	15	(+)?	+++
3	2,530	2,510	16	(+)	+++
4[2]	3,280	3,310	30[4]	(+)?	+
5	2,150	2,260	32	(+)	+++
6	3,200	3,020	41[4]	(+)	+++
7	2,200	2,320	56	(+)?	+++
8[3]	2,400	2,470	56	+	+++
9[3]	3,280	3,400	59[4]	+	+
10	2,530	2,530	60	(+)?	+++
11	2,570	2,830	61	(+)	++
12	2,150	2,250	61	(+)	+++
13	3,150	3,070	63	(+)?	+++
14[3]	3,500	3,500	65[4]	(+)?	+++

[1] (+)? = slight growth or distended by secretion only?
[2] = castration 33 to 35 days after hyp.ect.
[3] = castration 15 days before hyp.ect.
[4] = start of injections 27 to 35 days after hyp.ect.

after 32 and 61 days injections respectively, there may be a slight, stunted proliferation of ducts.

In the presence of anterior hypophysial tissue mammary gland growth is considerably more extensive and of a different type. This is revealed by the experiments of table 2 on incompletely hypophysectomized females. All the 15 glands studied from 14 to 68 days after the start of injections into 12 animals showed a development of the parenchyma similar to that shown in figs. 4 to 6 (exp. 6 and 10). A colourless secretion was found in most glands of this group but the distension was less than in those of table 1. It should be emphasized that a slight, but distinct proliferation of ducts was present in 3 glands of 2 rabbits with no other hypophysial tissue than a remnant of zona tuberalis in close contact with the infundibular tissue at and above the roof of the sella turcica.

The reaction of 8 and 7 mammary glands respectively of 5 completely and 5 incompletely hypophysectomized male rabbits was similar to that observed in the females of tables 1 and 2 re-

Table 2.

Incompletely hyp.ect. female rabbits castrated 3 to 4 weeks before hyp.ect. Start of injections of estradiol monobenzoate 18 to 39 days after hyp.ect.

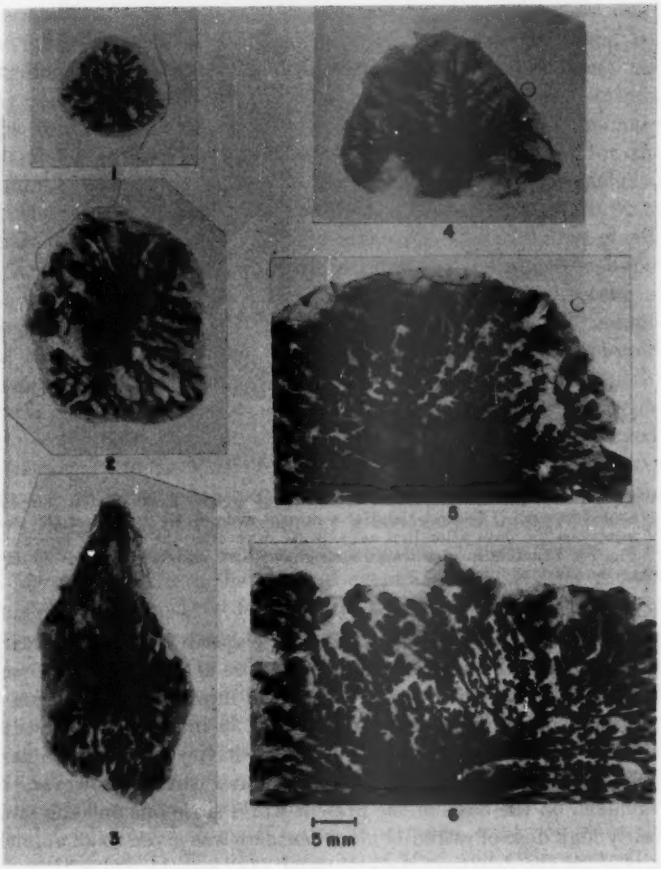
Exp.	Body weight at		Mammary glands examined after first injection			Remnant of pit. gl.
	hyp.ect.	exam. of m. gl.				
	g	g	days	growth	secretion	
1[1] ...	3,000	3,400	14	+++	—	+++
2[1] ...	2,500	3,500	22[3]	++	—	+++
3[1] ...	3,400	4,200	26[3]	+++	—	+++
4[1] ...	3,000	3,550	28	+++	—	+++
5[1] ...	2,150	3,420	29[2]	++	++	++[4]
6 ...	2,250	2,650	32[2]	+++	++	++
7 ...	4,200	3,400	41	++	++	(+)[4]
8 ...	2,250	2,650	56[2]	+++	++	++
9[1] ...	2,150	3,790	59[2]	++	++	++[4]
10 ...	2,250	3,000	61[2]	+++	+	++
11[1] ...	3,500	4,500	62[2]	+++	+	+++
12 ...	2,600	3,100	65	+++	++	++
13 ...	2,750	2,500	66	++	+	++
14 ...	3,250	3,150	67	++	+	+
15 ...	3,000	3,400	68	+++	—	++

[1] = castration 37 to 74 days after hyp.ect.
[2] = start of injections 1 to 6 days after hyp.ect.
[3] = start of injections 61 to 75 days after hyp.ect.
[4] = zona tuberalis only.

spectively. Since the glands examined before the start of injections were undeveloped in these two groups a proliferation was more easily recognized than in adult females in spite of distensions by secretion. Figs. 7 to 9 illustrate a typical reaction of the mammary gland of a completely hypophysectomized castrated male 27 and 57 days after the first injection of estradiol monobenzoate respectively. That the growth of the mammary gland is limited and stunted after a complete hypophysectomy is clearly seen when figs. 8 and 9 are compared with fig. 10 which shows part of a gland from a castrated male with an intact hypophysis 46 days after the first injection of estradiol monobenzoate.

Comments.

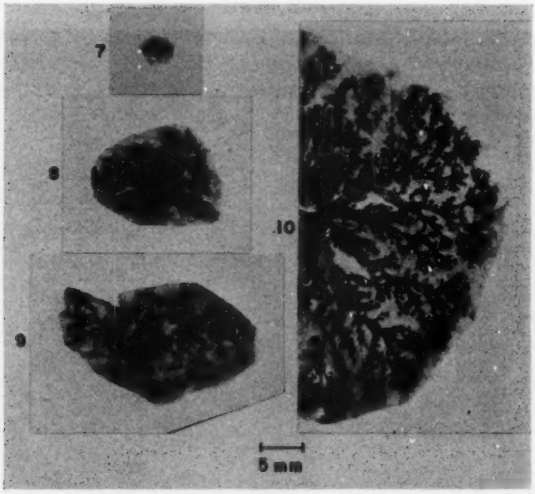
As indicated by the notes below the tables, the experiments were not grouped according to the intervals between castration, hypophysectomy and beginning of injections after these opera-



Whole mounts of mammary glands stained with gallocyanin chromalum and photographed with the same magnification. Figs. 5, 6 and 10 showing parts of the glands only. Uniform treatment with estradiol monobenzoate as described above.

Figs. 1—3. Reaction in a female rabbit (body weight 2,230 g) after complete hypophysectomy: 1) 27 days after castration. At hyp.ect., 2) 32 days after the start of injections (34 days after hyp.ect.), 3) 61 days after the start of injections.

Figs. 4—6. Reaction in a female rabbit (body weight 2,210 g) after incomplete hypophysectomy: 4) 28 days after castration. At incompl. hyp.ect., 5) 32 days after the start of injections (33 days after incompl. hyp.ect.), 6) 61 days after the start of injections.



Figs. 7—9. Reaction in a male rabbit (body weight 2,520 g) after complete hypophysectomy: 7) Control gland of a normal male. 8) 27 days after the start of injections (56 days after hyp. ect.). 9) 57 days after the start of injections.
Fig. 10. Reaction in a castrated male rabbit with intact hypophysis 46 days after the start of injections.

tions. In all cases enough time had elapsed for the mammary glands to atrophy and the type of response of the glandular tissue was similar irrespective of whether the injections were begun a few days after hypophysectomy or after an interval of more than a month. A gradual decline of the sensitivity of the mammary gland to estrogens during the first days cannot, however, be excluded on the basis of the present work, since one and the same fairly high dose of estradiol monobenzoate was given to all animals and since the degree of growth could not be measured with sufficient accuracy. The 100 μg dose of estradiol monobenzoate injected every 4th day was chosen because it had been shown to elicit a marked growth of the mammary glands of rabbits studied in previous work (JACOBSOHN 1949).

The experiments were not grouped according to body weight at hypophysectomy. As mentioned above each experimental gland was compared with a control taken from the same animal. The difference between the changes in the mammary glands of completely and incompletely hypophysectomized rabbits was the

same both in the immature and the adult rabbits studied. A detailed analysis using graded doses of estrogens might, however, reveal variations in the sensitivity of the mammary gland according to age and developmental stage at the start of injections. As pointed out by ASTWOOD et al., 1937, the nutritional state of the animals is of importance for the reactivity of the mammary gland to injected estrogens. This certainly holds true for the rabbit. 16 completely hypophysectomized rabbits, not included in the present material, that had a body weight loss of 10 % or more did not show any changes whatever of the mammary glands after estradiol monobenzoate injections. In incompletely hypophysectomized rabbits mammary gland growth may occur in spite of a poor nutritional state (table 2, exp. 7). The data presented show that the body weight of the completely hypophysectomized rabbits remained at about the same level during the experiments lasting 2 to 3 months. In the incompletely hypophysectomized animals a considerable increase was observed. With regard to the work of SAMUELS et al., 1941, on the mammary glands of force fed hypophysectomized rats, it does not seem justified to regard the mammary gland growth observed in the present experiments, as a simple function of body weight. But a factor (s) active in the chain of events leading to body growth might be assumed to facilitate also the stimulating action of estradiol monobenzoate on the mammary gland.

The observation of a very limited growth of the mammary glands of hypophysectomized rabbits injected with estradiol monobenzoate does not agree with those mentioned in the introduction (ASDELL and SEIDENSTEIN 1935, GOMEZ 1937, FREDRIKSON 1939). Although it seems difficult to find the cause of discrepant results in experiments of the kind where minor technical details may become of major importance, some points should be mentioned. 1) The investigators of 1935 and 1939 controlled the completeness of the hypophysectomy by means of serial sections through the contents of the sella turcica. In my experience during previous and the present work and in agreement with PICKFORD and VOGT (1951) it is necessary to examine also the brain tissues above the roof of the sella turcica. 2) ASDELL and SEIDENSTEIN compared the average weights of the mammary glands of 4 ovariectomized and 4 ovariectomized, hypophysectomized rabbits. Since it is difficult to isolate this gland from surrounding tissues and since the glands of the completely hypo-

physectomized rabbits in the present work were widely distended with secretion, the weight can hardly indicate mammary gland growth with reasonable accuracy. 3) As pointed out by RICHARDSON (1947) thin sections made from sample areas may be misleading in investigations of the growth and development of the mammary gland. Studies on whole mount preparations are more informative. FREDRIKSON examined only thin sections from a sample area.

With regard to the failure of GOMEZ and TURNER to obtain duct growth in the two completely hypophysectomized rabbits studied it should be said that no mention of body weight was made.

The findings reported agree with those of investigators who observed slight or no mammary gland growth after injections of ovarian hormones into hypophysectomized animals of other species. The observed stimulation of secretion and a slight proliferation seem to support the view that estrogens are acting directly on the mammary gland. But the possibility that the pars tuberalis of the median eminence which remains even after complete hypophysectomy plays some part in the stimulating action of estradiol on the mammary gland should be kept in mind (PICKFORD and VOGT 1951). The extensive proliferation which occurred in the incompletely hypophysectomized rabbits agrees with the well established proliferation promoted by ovarian hormones in rabbits with an intact pituitary gland and indicates the importance of the anterior pituitary gland for normal growth of the mammary gland.

The present observations allow a better understanding of the changes observed by JACOBSOHN (1949) on the mammary glands of rabbits with the hypophysial stalk transected.

Summary.

The effect of hypophysectomy on the reaction of the mammary gland to estradiol monobenzoate was studied in the rabbit. Observations made on 14 completely and 17 incompletely hypophysectomized, castrated males and females are presented.

In completely hypophysectomized rabbits injected with estradiol monobenzoate the mammary glands were distended with a colourless secretion and showed slight, stunted growth. After incomplete hypophysectomy extensive proliferation of ducts and slight secretion were found.

The observations support the view that anterior hypophysial as well as ovarian hormones are involved in the processes leading to growth and development of the mammary glands.

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The Assay of Hydrogen Peroxide in Small Quantities with Horse Radish Peroxidase as Catalyst.

By

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The use of a peroxidase as a catalyst in the reaction

$$AH_2 + H_2O_2 \longrightarrow A + 2\ H_2O$$

(AH_2 = oxidizable, hydrogen donating substance) (A = dye or dyeforming product)

offers some definite advantages when H_2O_2 is to be determined especially in small quantities:

a) Specificity. The use of leucodyes is possible but inconvenient because of their oxidizability by O_2.

b) Peroxidases and H_2O_2 form rapidly complexes of very small dissociation constants (review by CHANCE 1951). Minute quantities of the peroxide in a biological fluid can thus be caught, provided that the peroxidase is present in sufficient concentration.

c) Reaction conditions, fitting to the requirements of an actual situation, can be selected within fairly wide limits, since the velocities of the reactions between (peroxide-peroxidase) and the different oxidizable substances vary greatly (CHANCE 1951), and since different colours are obtained from different hydrogen donors. JAYLE (1939) and JOSLYN (1949) have listed numerous substances, oxidized by H_2O_2 + peroxidase.

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The effect of catalase, eventually present in the peroxide-containing fluid, is largely reduced in acid acetate buffer (BONNICHSEN, CHANCE, and THEORELL 1947) or by high peroxidase concentration.

THURLOW (1925) used nitrite plus milk, which contains a peroxidase, to demonstrate the formation of H_2O_2 during the action of xanthine oxidase. MAIN & SHINN (1939) determined H_2O_2 in quantities of 6—30 μg per ml by means of a crude potato extract and *o*-tolidine. PATRICK & WAGNER (1949), HUMPOLETZ (1949) and FREYTAG (1950) determined H_2O_2 by means of iodide, titanic salt and lead sulphide respectively, the sensitivities reaching 0.01—20 μg per ml.

With access to pure peroxidase it seemed possible that even smaller amounts of H_2O_2 could be assayed, and that the procedure could be arranged to make it applicable to clinical problems, *e. g.* in dermatology and bacteriology.

Material.

Crystallized horse radish peroxidase (HRP) (THEORELL 1942) was used.

d-amino acid oxidase apoprotein was prepared according to NEGELEIN & BRÖMEL (1939) (first five steps). It was dialyzed against distilled water until free from sulphate ions. One tenth milliliter of the stock solution, diluted to 3.0 ml, gave $D_{280\ m\mu} = 0.210$.

Flavine adenine nucleotide (FAD) was prepared according to DIMAND, SANADI, and HUENNEKENS (1952). These authors give the purity of the product as 10 %, which would mean that $5 - 10 \times 10^{-9}$ moles of FAD (M = 785) were used in the reaction mixture of 3 ml.

Ordinary distilled water and a. g. reagents were used. Solvents were tested for peroxide immediately before used. The experiments were made at room temperature. Light absorptions were determined with a Beckman DU spectrophotometer, 1-cm cells.

Results.

A number of oxidizable substances were tried. The benzidine blue colour is favourable, since it gave, at acid reaction, the most intensive colour and since maximal intensity was reached within a few seconds. The benzidine blue is unstable, but the fading is slow ($t_{1/2} \sim 3$ min., pH 3.7) and backward extrapolation to the apparent zero time value is easy. This is linearily proportional to the added amount of peroxide (Fig. 1). About 0.5 μg can be assayed in this way.

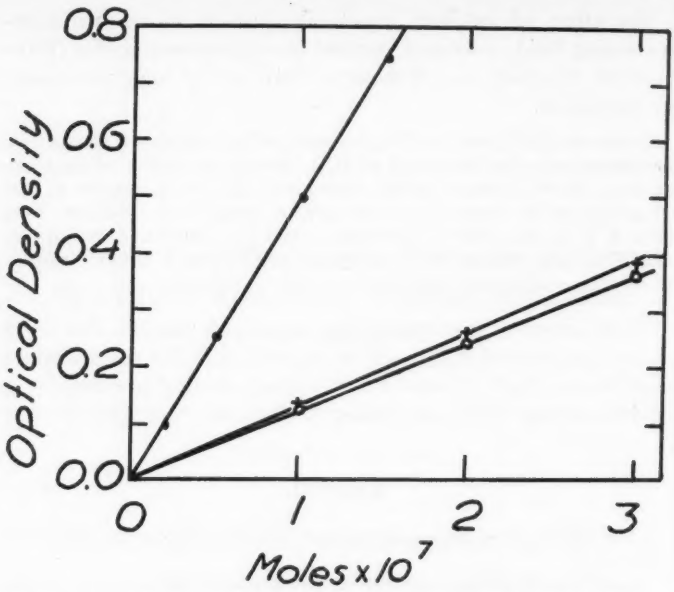

Fig. 1. Optical densities of dyes, produced by the action of HRP + H_2O_2.

● 0.10 ml 4 m*M* alcoholic benzidine solution + 2.0 ml 0.2 *M* acetate buffer pH 3.7 + 0.2 ml 12 μM HRP + H_2O_2 + aq. ad 3.0 ml Apparent zero time densities at 560 mμ plotted against added amounts of H_2O_2.

\+ Reference curve with guaiacol-benzidine mixture. 0.10 ml of (4 : 1 mixture of m*M* alcoholic solutions of guaiacol and benzidine) + 0.30 ml *M*/15 pyrophosphate buffer pH 8.3 + 0.10 ml. 12 μM HRP + H_2O_2 + aq. ad 3.0 ml. Densities at 510 mμ plotted against added amounts of H_2O_2.

○ Densities of butanol extracts from the reaction with d-amino acid oxidase, plotted against added amounts of d-alanine.
Butanol extracts made up to three ml before measured.

The following semiquantitative arrangement is more sensitive. Amberlite XE-97 (200—400 mesh) was suspended in 0.1 *M* acetate buffer of pH 3.7 and poured on a sintered funnel to give a layer of 2—3 mm thickness. The excess of buffer was removed by gentle suction, leaving a moist surface. Areas of 5 mm diameter were marked with a glass tube. While gentle suction was applied 0.05 ml of each of 0.1 % alcoholic benzidine solution and 0.4—0.6 m*M* HRP in water were put on the spots. The liquids were applied from pipettes, held perpendicularly against the surface. The H_2O_2-containing solution was then applied in the same way. It was found that 10^{-10} moles of H_2O_2 (0.003 μg) in 2 ml

Table 1.

Semiquantitative determination of H_2O_2 in bacteriological media. Two ml of undiluted solutions and dilutions 1 : 1, 1 : 5, 1 : 10, 1 : 50, and 1 : 100 were used. Manipulations performed in diffuse daylight. All undiluted solutions gave negative tests for H_2O_2 immediately and 17 hours after the end of the experiments when kept in darkness.

Solution	Solution kept in	Time min.	Diln. giving colour intensity = $5 \times 10^{-6} M$ H_2O_2
2 % acid digested casein in water	Darkness	0—60	0 colour
—»—	Diffuse daylight	20 60	1 : 1 1 : 10
—»—	Darkness + O_2-bubbling	20—60	1 : 5
—»—	Diff. daylight + O_2-bubbling	20—60	1 : 10
—»—	UV-light from Wood's lamp in 30 cm distance	1 5 10—15	1 : 5 1 : 10 1 : 50
1 % cryst. serum albumin in water	—»—	1— 5 10—15	1 : 1 1 : 5
2 % glucose in water	—»—	20	0 colour
10 μM cysteine. HCl in water (pH 3.7)	Darkness	15	1 : 50

gave a distinctly blue spot, whereas the half of this quantity did not. A five times larger amount in 2 ml gave a considerably stronger colour. The colour faded in a few minutes. The concentration of H_2O_2 in a solution of unknown strength could be estimated by diluting the solution with water until 2 ml gave a spot of roughly equal intensity as 2 ml of 5×10^{-8} M H_2O_2. The formation of peroxide in some commonly used bacteriological media was tested with this spot method (Table 1).

Hydrogen peroxide, formed during the action of a flavine enzyme (d-amino acid oxidase) was determined in the following way. A mixture of guaiacol and benzidine in the molar ratio of 4 : 1 as hydrogen donor gave a red-violet colour when oxidized. Either substance alone was unsatisfactory. The intensity of the

colour and the velocity of its formation were nearly the same at pH 3.8 and 8.3. Maximal light absorption (510 mμ) was reached within two minutes, after which time the colour faded slowly (one hour) to leave a yellow residue. The red-violet substance could be quantitatively extracted by 3 × 1 ml butanol in which it was stable for hours. This was done after two minutes in the determination of the reference curve.

The following reaction mixture was used.

Apoprotein	0.10 ml
FAD (1 mg/ml)	0.06 »
Pyrophosphate buffer *M*/15, pH 8.3	0.30 »
Guaiacol-benzidine solution	0.15 »
HRP (12 μM)	0.20 »
d-alanine (m*M*)	0.0—0.3 ml
Water to	3.0 ml

Millimolar solutions of guaiacol and benzidine in alcohol were mixed in the volume ratio of 4 : 1 immediately before used. The d-alanine was added as dl-alanine (stock solution 8.9 mg/50 ml) in water. Apoprotein, FAD, and buffer were mixed 40 min. before the other reactants were added. The stop watch was started when the last component, alanine, was pipetted. The red-violet colour reached its maximum after 12 min. and was then extracted with 3 × 1 ml of butanol. The result is given in Fig. 1. As an average 94 % of the light absorption was found calculated from added d-alanine and assuming 100 % oxidation. If HRP was not present at the beginning but added 5 min. after the alanine, only one fifth of the colour intensity was obtained.

Summary.

1. Hydrogen peroxide can be assayed colorimetrically by means of horse radish peroxidase + a hydrogen donor, which after oxidation reacts to give a coloured compound. The reaction conditions can be modified to fit the actual situation.

2. In a semi-quantitative arrangement 10^{-10} moles of peroxide (0.003 μg) in a few ml of solution can be determined.

3. 94 % of the calculated amount of hydrogen peroxide were found in the oxidation of d-alanine by means of d-amino acid oxidase.

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A Note on the Uptake of Radioactive Phosphate in the Skeleton of Rachitic Rats.

By

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In early experiments involving the use of radioactive phosphate (P^{32}) COHN and GREENBERG (1939) succeeded in demonstrating that the uptake of P^{32} was much lower in rachitic rats than in control animals. After administration of vitamin D, there was at the end of three days an increased uptake of P^{32} in the femurs of the rachitic rats. These experiments have been confirmed by many others (*inter alios* MORGAREIDGE and MANLY 1939, CLAASSEN and WÖSTMANN 1954). From investigations on the metabolism of labelled calcium (Ca^{45}) COPP, HAMILTON, JONES, THOMPSON and CRAMER (1951) concluded that the initial rapid uptake of the labelled substance in the skeleton, *i. e.* the uptake due to ionic exchange, is of the same order of magnitude both in rachitic and control animals. In the rachitic animals there was, however, a rapid loss of the Ca^{45} due to lack of new deposition of mineral salts.

Earlier experiments (ZETTERSTRÖM 1952) have shown that the inorganic portion of bone consists of different fractions with varying solubility in salt solutions. The fraction which is extractable with saturated ammonium sulphate of pH 7.5 has a much higher rate of renewal than the remaining one. The P^{32} initially taken up in the skeleton was found to be mainly deposited in lowly mineralized areas of the compact bone, *i. e.* in young Haversian systems and in the endosteal and periosteal layers (ENG-

FELDT, ENGSTRÖM and ZETTERSTRÖM 1952). It can thus be concluded that the fraction of the bone minerals which is extractable with saturated ammonium sulphate, is localized at not fully calcified structures. When the mineralization in a Haversian system increases, there is a successive decrease of the solubility in ammonium sulphate at the same time as the ionic exchange is declining. Thus, the rate of uptake of labelled phosphate in the fraction insoluble in saturated ammonium sulphate, can be used as an index of the accretion of mineral salts.

In this communication experiments concerning the uptake of P^{32} in the ammonium-sulphate and remaining fractions of bone minerals in rachitic rats and control animals will be reported. Some of the rachitic rats were treated with 30 I. U. of vitamin D_2 three days prior to the administration of P^{32}.

Litter-mate male rats, 3 weeks old, were placed on a rachitogenic diet (STEENBOCK and BLACK 1925) for four weeks. The animals were then injected intraperitoneally with 0.10 mC radioactive phosphate, *i. e.* $Na_2HP^{32}O_4$, containing negligible amounts of phosphate. After the lapse of the desired time interval, the animals were killed by decapitation. Blood was collected from the carotid arteries and deproteinized in ice-cold 15 per cent trichloracetic acid. The specific activity, *i. e.* number of impulses/microg. phosphate, of orthophosphate was then determined directly (ERNSTER, ZETTERSTRÖM and LINDBERG 1950). Bone specimens (femur and tibia from the same leg) were removed immediately after death, and epiphyseal and diaphyseal bone was separated. The bone specimens were then extracted with ammonium sulphate and 15 per cent trichloracetic acid as earlier described (ZETTERSTRÖM 1952). With this method the bone minerals were completely extracted. The fraction insoluble in ammonium sulphate but extracted by trichloracetic acid, will be referred to as the remaining one. In the separate experiments the weight of the bone powder extracted was about 150 mg. 10 ml of saturated ammonium sulphate was used for the first extraction.

There was no significant difference between rachitic and normal rats with regard to the amount of bone minerals soluble in the volume of saturated ammonium sulphate used. In epiphyseal bone about 30 per cent of the minerals was dissolved in this fraction, in diaphyseal bone only 15—20 per cent was extracted.

Tables 1 and 2 clearly demonstrate, in rachitic as well as control animals, the same high initial uptake of activity in the fraction extractable with ammonium sulphate, whether the bone is epiphyseal or diaphyseal. During the time of study, there is no significant difference in the labelling of this fraction between the three groups of animals. In the remaining fraction, however, the

Table 1.

Relative specific activity in epiphyseal bone in normal rats, rachitic rats, and rachitic rats treated with vitamin D.

Specific activity of orthophosphate in blood = 100. Each value represents the mean of the determinations in three animals. Radioactivity was measured under a counter after drying an aliquot of the extract on an aluminium dish.

Interval after injection of P^{32}	Relative Specific Activity					
	$(H_4N)_2SO_4$ fraction			Remaining fraction		
	Controls	Rachitic animals	Rachitic animals treated with vitamin D	Controls	Rachitic animals	Rachitic animals treated with vitamin D
1.5 hours .	27.5	29	25	2.1	0.8	1.4
3 hours...	35	37	30	3.4	1.5	3.5
6 hours...	45	42	41	5.0	1.4	5.8

Table 2.

Relative specific activity in diaphyseal bone in normal rats, rachitic rats, and rachitic rats treated with vitamin D.

Specific activity of orthophosphate in blood = 100. Each value represents the mean of the determinations in three animals.

Interval after injection of P^{32}	Relative Specific Activity					
	$(H_4N)_2SO_4$ fraction			Remaining fraction		
	Controls	Rachitic animals	Rachitic animals treated with vitamin D	Controls	Rachitic animals	Rachitic animals treated with vitamin D
1.5 hours .	13	14	12	0.77	0.58	0.76
3 hours...	16	14	15	1.4	0.57	1.8
6 hours...	22	15	23	2.8	0.70	3.6

labelling was much lower in the rachitic animals than in the controls and those treated with vitamin D. The result was the same in epiphyseal as well as diaphyseal bone. The difference in the uptake tends to increase as the interval after the administration of P^{32} is extended. This observation is in accordance with the fact that the new deposition of mineral salts has ceased in rickets. The uptake of the bone minerals in the remaining fraction is the same in rachitic rats treated with vitamin D as in the controls.

In view of the findings of CLAASSEN and WÖSTMANN it could be expected that the labelling would be higher in the treated animals than in the normal ones. The lack of such a difference might be due to the fact that we have only performed short-time experiments.

The results obtained are as expected. The ionic exchange in poorly calcified areas, *i. e.* in the fraction soluble in ammonium sulphate, is the same in rickets as under normal conditions. These results have been confirmed by autoradiographic studies of the distribution of radioactive phosphate in short-time experiments with the compact bones of rachitic puppies (ENGFELDT and ZETTERSTRÖM). In rickets the new deposition of mineral salts is low, and consequently the uptake of radioactivity in the fraction insoluble in saturated ammonium sulphate is very low. The results show that by determining the uptake of radioactivity in the fraction which is not extractable with ammonium sulphate, it is possible to collect information concerning the rate of accretion of bone minerals. In this fraction the uptake due to ionic exchange seems to be very low.

Summary.

The bone tissue from rachitic rats were examined using tracer technique. The rats were injected with P^{32} and killed 1.5 to 6 hours after the injection. The relative specific activity of different fractions of the bone salts was determined. It could be shown that the uptake of isotope in the rapidly labelled fraction was the same as in normal animals. In the remaining fraction, however, the uptake of activity was much less in the rachitic animals than in the normal.

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Secretory and Vascular Effects of Various Drugs Injected into the Submaxillary Duct.

By

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Injection of chemical agents into the submaxillary duct in order to affect the activity of the salivary gland has been tried by some early investigators. GIANNUZZI (1864) found that hydrochloric acid or sodium carbonate, administered in this way, abolished the secretory effect of chorda stimulation in dogs. HEIDENHAIN (1874), although himself using injection of quinine for the same purpose, considered this method of application unsuitable and the effects observed due to mechanical injury to the nerve endings in the gland. LANGLEY (1878), however, made use of the method in cats in his studies on the mutual antagonism between pilocarpine and atropine. In order to produce vasoconstriction in the submaxillary gland of dogs BOTTAZZI et al. (1908) injected adrenaline into the secretory duct.

Methods.

Twenty-five cats and 3 dogs under chloralose anaesthesia (80—90 mg/kg) were used. In 14 of the cats and 2 of the dogs the chorda tympani or the chorda-lingual nerve had been cut some time in advance (10 days—9 weeks) in order to sensitize the gland to chemical agents (for references see EMMELIN and MUREN 1951). The arterial pressure was recorded in the femoral artery. The submaxillary ducts were cannulated on both sides using cannulae which delivered about 35 drops out of one cc of distilled water. Injections were made either into a femoral vein or into the submaxillary duct through a fine rubber tubing connected to the

submaxillary cannula. Before the injection the tubing was obstructed by a clip; the fluid was injected during 5 seconds and the clip removed 5 seconds after the end of the injection. Drops of saliva falling from the tip of the cannula were recorded on the smoked drum using a signal. In six of the cats the blood flow through the submaxillary gland was in addition recorded by an ordinate recorder operated by a phototube counter (CLEMENTZ and RYBERG 1949). In this way the drops of blood were recorded that fell from a cannula inserted into the external jugular vein, all branches which did not carry blood from the gland having been tied and the animal heparinized.

For the injections into the submaxillary ducts the following precautions were found to be important:

1. Before the injection of a drug a control injection of saline solution should always be made and the effects obtained with drugs compared with those of saline solution. As will be shown below, saline solution alone may cause secretory and vascular effects in the glands.

2. The volume injected should not be big, 0.1 or at the most 0.2 cc in a cat and 0.3 cc in a dog. When bigger doses are given the gland is found to give a reduced response to drugs injected into the duct or intravenously. After injection of 0.1 cc of Indian ink the whole surface of the gland was found to be stained, whereas half the volume was insufficient for this purpose.

3. The time interval between two injections should not be too small, since the effect of drugs is otherwise found to diminish. In our experiments an interval of 15 minutes was found to be convenient.

4. It is necessary to ascertain that a drug is not partly left in the cannula and pressed into the gland during a subsequent injection. If a drug has not produced a flow of saliva big enough to remove all of the substance from the cannula a control dose of saline solution should be injected.

Results.

1. Secretory Effects in Cats.

Saline solution. In the first experiment saline solution was injected into the submaxillary duct as a control. The result was a pronounced and long-lasting secretion of saliva. The injection of 0.1 cc of saline solution caused a flow of 43 drops of saliva during 15 minutes from the right gland, the chorda of which had been cut 26 days earlier, and 7 drops during 10 minutes from the left, normally innervated gland. On increasing the dose to 0.2 cc, 43 drops were secreted from the right and 19 from the left gland. In the course of the following investigation, however, such an effect was only rarely encountered. A marked secretory effect of saline solution could be elicited in 3 experiments only, out of 25. In the remaining cases a volume of fluid not greater than that

injected was obtained. The mechanism through which this secretion was brought about could therefore not be fully analysed. The following observations, were, however, made. The effect was obtained after the acute severance of the chorda-lingual nerve and the sympathetic trunk in the neck. The response to saline solution was greater after previous section of the chorda, as illustrated in the example given above. This was the case even in most of those instances in which the volume collected was smaller than that given. The secretory response was not affected by an intravenous dose of dihydroergotamine (1 mg) which rendered a moderate amount of adrenaline ineffective. It disappeared, on the other hand, after atropine, 1 mg intravenously, or 0.2 mg injected into the submaxillary duct. It seemed to disappear, likewise, after an intravenous injection of 5 mg/kg of hexamethonium.

Adrenaline. After injection of adrenaline into the duct a secretion was obtained which increased with the dose given. The threshold dose was of the order of 0.2 μg. The supersensitivity to adrenaline administered intravenously which is brought about by previous section of the chorda could be demonstrated when the drug was given into the duct also, the threshold dose in a fully sensitized gland being about 0.05 μg or less. In one particularly sensitive gland it was found to be 0.005 μg. A maximal effect was obtained with 5—20 μg in a normal gland and with 0.5—1 μg in a sensitized gland. With bigger doses the responses tended to decrease. The duration of the secretion varied between 1 and 5—6 minutes, for equal doses being greater on the sensitized than on the normal side.

Even big doses of adrenaline given into the duct were devoid of pressor action and there was never any flow of saliva from the contralateral gland. In one cat, for instance, 20 μg of adrenaline were given into one duct. It caused no blood pressure effect, and no secretion from the sensitized gland of the other side, which responded to 0.05 μg injected into its duct or 1 μg/kg given intravenously.

Fig. 1 gives an experiment with adrenaline. The gland the chorda of which had been severed 13 days previously gave a much bigger response to adrenaline, given intravenously or into the duct, than the contralateral gland. For instance, 0.5 μg of adrenaline, injected into the duct, produced on the decentralized side a much bigger response than 10 μg injected on the control side. Much

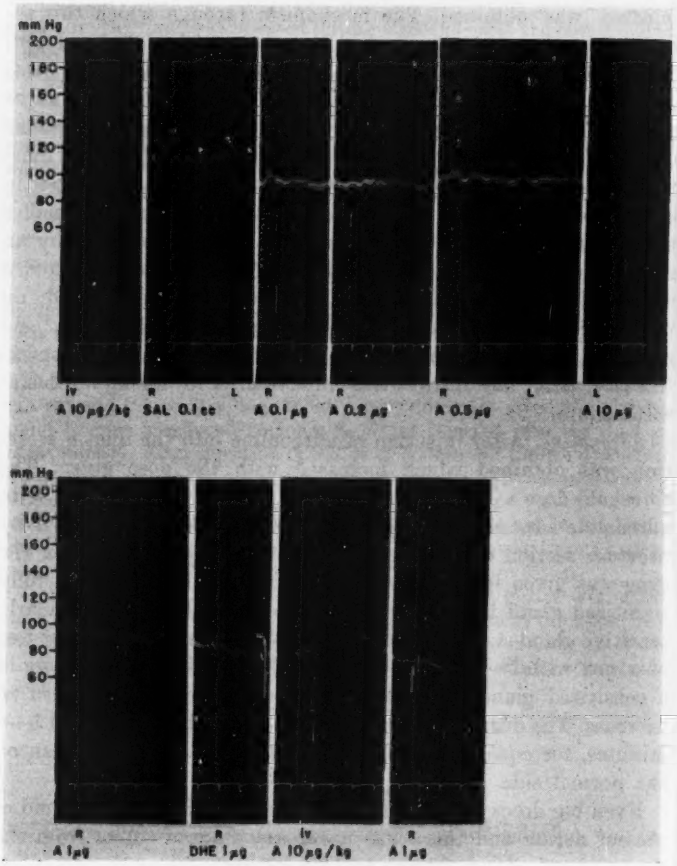

Fig. 1. Cat, 3.3 kg. Right chorda cut two weeks earlier. Records from above: blood pressure, secretion from right gland, from left gland, time in minutes, signal. A = adrenaline, DHE = dihydroergotamine. Injections intravenously (i. v.) or into the right (R) or left (L) duct in a volume of 0.1 cc.

smaller doses were required when the drug was injected into the duct than when it was given intravenously. 0.5 μg of adrenaline into the duct produced, for instance, an effect comparable to that caused by 10 μg/kg (33 μg) intravenously. Even a dose as big as 10 μg injected into the duct did not produce any secretion from the contralateral, sensitized gland, and no pressor effect.

Noradrenaline. Similar effects were obtained with noradrenaline. Somewhat bigger doses of this drug were required than with adrenaline.

Dihydroergotamine. The antagonistic effect of dihydroergotamine towards adrenaline could be demonstrated when the drug was given into the duct. The smallest effective dose of dihydroergotamine was 0.1—0.5 μg, whereas 1 μg had a strong effect. As can be seen in fig. 1 this dose markedly inhibited the effect of adrenaline injected intravenously or into the duct. The effect of dihydroergotamine remained local; the pressor effect of adrenaline given intravenously, or the secretory effect of the contralateral gland was not affected. This was the case even with 20 μg of dihydroergotamine.

Cocaine. This drug was found to exert two of its characteristic actions when injected into the submaxillary duct. The secretory effect of noradrenaline was found to be augmented just as is the case when the drugs are given intravenously (EMMELIN and MUREN 1951). A dose of 1 mg of cocaine injected into the duct had this sensitizing effect. This dose had in addition a paralyzing action on the secretory nerve fibres; after its injection neither chorda nor sympathetic stimulation gave rise to any salivary secretion, whereas the secretory effect of acetylcholine remained unaffected. As much as 10 mg of cocaine was required to diminish the response to acetylcholine. This big dose sometimes had a general effect, causing a slight rise in blood pressure.

The effects of cocaine, given into the duct, were found to be transient, the responses to noradrenaline decreasing and those to nerve stimulation returning. A second dose of cocaine then caused similar effects as the first one.

Acetylcholine. Acetylcholine given into the duct caused a secretion of short duration. The threshold dose was found to be 0.5—1 μg. After previous section of the chorda it was found to be lowered to 0.1—0.5 μg. The injection of 5 μg into the duct was in some instances, but not always, followed by a small fall in blood pressure. Even with 100 μg there was only a moderate depression of the general pressure and no secretion from the contralateral gland.

Pilocarpine. The secretory effect of this drug can be studied in the experiment of fig. 2. As little as 0.2 μg of pilocarpine hydrochloride caused a secretion from the gland, the chorda of which had been cut in advance. With 0.5 μg, injected into both ducts in the experiment, a response was obtained from the normal gland

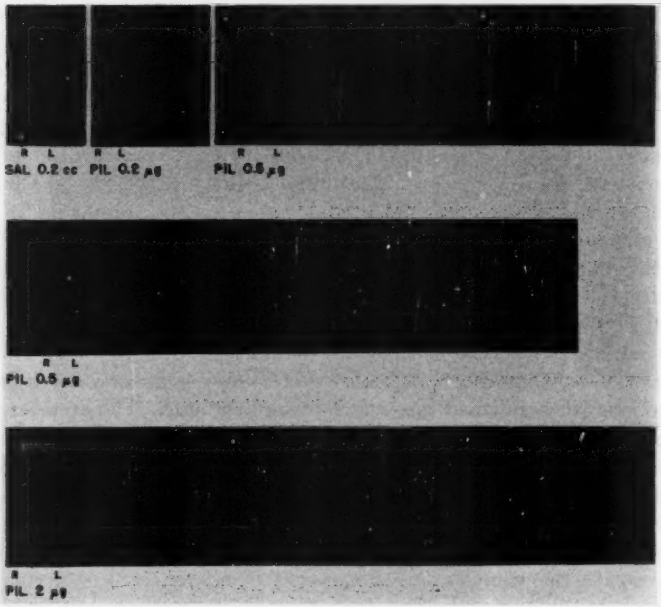

Fig. 2. Cat, 4.0 kg. Right chorda cut 18 days previously. Records from above: secretion from the right gland, from left gland, time in minutes, signal. Pil = pilocarpine hydrochloride, injected in 0.2 cc into the ducts. SAL = saline solution.

also, and the effect on the supersensitive gland was pronounced. It was, however, found to be transient, and on repetition of the injection a similar response was elicited. Bigger doses (the effects of which are not shown in the figure) caused a secretion of greater rate and duration, but even after 200 μg it came to an end within an hour. A dose of 20 μg had a local effect only. 200 μg on the other hand, gave a fall in blood pressure, and ten minutes after the injection a slow secretion started from the contralateral gland. This was the gland sensitized by denervation and responding to 0.2 μg of pilocarpine injected into the duct. When the effect of the big dose had worn off, the sensitivity of the gland to intravenously administered pilocarpine was tested. The gland was found to respond to a dose of about 30 μg. An amount of pilocarpine of at least this order of magnitude had obviously escaped into the general circulation after the injection of 200 μg into the

duct. For comparison it may be added that the normally innervated gland, responding to 0.5 μg on injection into the duct, did not start secreting on intravenous doses smaller than about 90 μg of pilocarpine.

Eserine. Already 0.1 μg of eserine sulphate given into the duct was found slightly to increase the effect of acetylcholine administered intravenously or into the duct. With 1 μg the effect was marked. The secretory effect of a moderate dose of acetylcholine was about doubled, and stimulation of the chorda caused a prolonged secretion. After 1—10 μg of eserine a flow of saliva started, after a latency of 1—2 minutes. In one experiment, for instance, 10 μg caused a flow of 15 drops of saliva during about 35 minutes from a gland, sensitized by chorda section, and the same dose given to the contralateral gland gave 3 drops during about 10 minutes from this gland. The injection of 10 μg of eserine into the duct did not affect the blood pressure, but when the chorda was then stimulated a depressor effect was noted in one case. 50—100 μg of eserine into the duct caused a secretion at maximal rate and about 10 minutes after the injection the blood pressure began to fall, either due to absorbed eserine or a great output of acetylcholine from the eserinized gland. Even with these big doses the secretory effect was transient and the injection could be repeated with similar result.

Atropine. The secretory effect of acetylcholine was found to be considerably reduced by the injection of 1 μg of atropine sulphate into the salivary duct. For instance, 1 μg of acetylcholine given into the duct caused a flow of 6 drops of saliva before, and no secretion after this dose. 10—15 μg of atropine rendered chorda stimulation completely ineffective on the secretory cells and, likewise, pilocarpine in a dose of 200 μg, *i. e.* about 400 times the threshold dose. With still greater doses of pilocarpine a small secretion could be elicited. Moderate doses of atropine did not affect the response to sympathetic stimulation. The action of atropine was found to be reversible and it was observed that the duration of action could be considerably shortened by chorda stimulation.

The effect even of 200 μg of atropine was restricted to the gland into the duct of which the injection had been made; the depressor action of an intravenous dose of acetylcholine was not diminished, nor was the acetylcholine sensitivity of the contralateral gland reduced.

Hexamethonium. 1 mg of hexamethonium chloride gave a small but definite diminution of the response to chorda stimulation on the side injected. The blood pressure and the contralateral gland were not affected. 10 mg of the drug given into the duct caused a fall in blood pressure and reduced the effect of chorda or sympathetic stimulation on both sides to some degree. Particularly diminished, although by no means abolished was the response to chorda stimulation of the injected gland. The secretory effect of acetylcholine or adrenaline was not altered. When the effect of the hexamethonium had worn off, 10 mg of the drug were injected intravenously. Stimulation of the chorda or the sympathetic trunk was now ineffective, whereas acetylcholine retained its previous activity.

Nicotine. Nicotine injected into the submaxillary duct was found to cause a flow of saliva, which was greater after previous section of the chorda. A small effect could be elicited with 10 μg of nicotine bitartrate. With 100 μg of the drug 17 drops were obtained in one hour from a gland, the chorda of which had been cut three weeks earlier, and 3 drops in some minutes from the contralateral, normal gland. These effects could be repeated with fresh injections. The flow ceased after the injection of atropine intravenously. The effect was, likewise, abolished by the intravenous injection of 5 mg/kg of hexamethonium chloride.

The secretion elicited by nicotine was fairly slow and not at all as rapid as that obtained by chorda stimulation. A nicotine bitartrate dose of 1 mg did not cause a quicker secretion than did 100 μg from the supersensitive gland. Even after the bigger dose the secretion caused by chorda stimulation was not markedly reduced. No effects on blood pressure or respiration were observed after injection of 1 mg of the nicotine salt into the duct.

2. Vascular Effects in Cats.

In all the six experiments saline solution, injected into the duct, was found to increase the flow of blood through the gland. In none of these cases a definite secretory response was obtained. The vascular effect was evanescent, lasting for a few seconds only after 0.05 cc, for about half a minute after 0.1 cc of saline solution. In some cases it was almost as great as that obtained on stimulation of the chorda. Saline solution of body temperature, plasma or saliva produced equal effects. The effect remained after

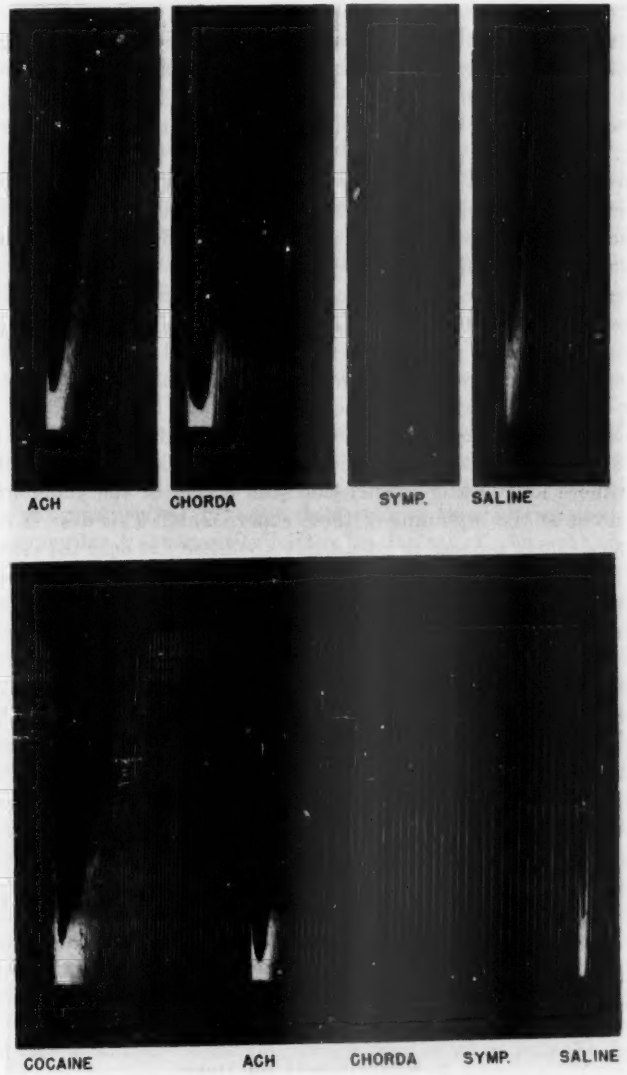

Fig. 3. Cat, 2.1 kg. Records from above: secretion from the right gland, blood flow through right gland, signal, time in minutes. ACH = 5 μg acetylcholine injected in 0.1 cc saline into right duct. The dose of cocaine, given into this duct, was 10 mg and the volume of saline 0.1 cc.

atropine in a dose which abolished the secretory effect of chorda stimulation, and after intravenous administration of hexamethonium chloride, 5 mg/kg, which completely anulled the secretory and vascular effects of chorda stimulation. Nor did cocaine, injected into the duct abolish the response to saline solution (fig. 3).

Acetylcholine in a dose of 1—5 μg produced a marked vasodilatation. Pilocarpine, 10 μg, had a similar although more long-lasting effect, which was abolished by atropine. With adrenaline, 1—5 μg, a vasoconstriction, sometimes followed by a vasodilatation was observed. A bigger dose of adrenaline, 10 μg, was found to cause a constriction which could last for as much as one hour or more. This finding may explain the observation that secretory effects of various agents were often much diminished after injection of a big dose of adrenaline into the duct.

Cocaine in a dose of 1 mg injected into the duct was found to cause a small dilatation of short duration, followed by a slight but more long-lasting constriction (the nerves of the gland had been cut at the beginning of these experiments). This dose of cocaine strongly reduced or completely abolished not only the secretory but also the vascular effects of chorda of sympathetic stimulation, whereas the responses to acetylcholine were not changed. 10 mg of cocaine produced similar vascular effects as 1 mg, only they were more pronounced with the bigger dose.

Some of the effects of cocaine, 10 mg, can be studied in the experiment of fig. 3. The figure shows the increased flow of blood through the gland after the injection of saline solution. The secretory and vascular effects of acetylcholine, injected into the duct, and of chorda and sympathetic stimulation are, likewise, illustrated. It can be seen that cocaine completely abolished secretion and vasodilatation caused by chorda stimulation, and secretion and vasoconstriction elicited by sympathetic stimulation. Acetylcholine, on the other hand, retained its vasodilator action, and the secretory effect was only moderately diminished by this big dose of cocaine. Injection of saline solution still increased the flow of blood through the gland.

3. Experiments on Dogs.

Injections into the submaxillary ducts were made in three dogs, earlier used for other observations. Secretion could be elicited by

injection of acetylcholine, 0.3 μg causing a just detectable response, 3 μg 11 drops in one case. Pilocarpine, 0.3—1 μg, caused a flow of saliva from a normal gland; a maximal rate of secretion from a denervated gland was obtained after injection of 3 μg. With adrenaline a secretion was elicited after injection of 3—15 μg; after previous section of the chorda the gland responded to 0.3 μg. In one dog histamine, 3, 30 and 300 μg, were injected into the duct; no secretion could be observed. In one case 30 mg of sodium cyanide was given through the duct, without any effects on the arterial pressure or the respiration; the same dose given by vein proved to be lethal.

Discussion.

Drugs which stimulate or decrease the secretion of saliva when given by vein have been found to have a similar effect if injected into the submaxillary duct. Much smaller doses are required if the drugs are given into the duct than if they are injected intravenously. A supersensitivity to chemical agents, brought about by previous section of the chorda tympani can be demonstrated for this mode of administration also. It may perhaps not be justified to conclude that the various drugs exert their action on the secretory cells from the part of the cells facing the lumen of the ducts. Very likely they penetrate into the tissue spaces and may act on the cells as if brought to the gland by the blood stream. The fact that they obviously may reach the walls of all the small vessels causing *i. e.* a vasodilatation as pronounced as that elicited by chorda stimulation seems to speak in favour of this conception. It is, however, remarkable that even fairly big doses of the drugs are not absorbed into the blood stream, in amounts big enough to cause general effects. The mode of administering drugs, studied in this investigation, may therefore offer certain advantages from a methodological point of view. The contralateral gland is not reached and may thus serve as a control. General effects of the drugs for instance on the circulation do not interfere with the experiment (a lowered blood pressure, for instance, may considerably decrease the rate of salivary secretion). Drugs may be given into the duct to reach a concentration in the gland which could not be attained by the intravenous route. The preparation may thus offer the advantages of an isolated gland, but with normal innervation

and blood supply. In this way it was possible to give cocaine in amounts big enough to paralyze the fibres of the autonomic secretory and vasomotor fibres in the gland. The choline esterases of the gland can, very likely, be more completely paralyzed by eserine when this way of administration is made use of; even with the biggest intravenous doses of eserine tolerated the inactivation of these enzymes is far from complete (EMMELIN and MACINTOSH 1948). The same is probably true for other enzyme inhibitors such as cyanides. The analysis of the mode of action of a drug on the gland may be easier if its effects remain local on the gland. Histamine, for instance, may cause a flow of saliva when given intravenously. This effect may, in part at least, be due to adrenaline, released from the adrenal medulla. In our experiment on a dog histamine did not cause salivary secretion when injected into the duct. A further advantage of this way of administering drugs may be that the actions are more transient. Drugs such as pilocarpine, eserine, atropine and dihydroergotamine which often act for the whole of an acute experiment when given intravenously had in our experiments a relatively short duration of action when injected into the duct, and the effects of repeated doses of the drugs could be studied.

The duration of action of *i. e.* atropine was found to be shortened by chorda stimulation. This may be due to a mutual antagonism between atropine and liberated acetylcholine, as studied for atropine and pilocarpine by HEIDENHAIN (1874) and LANGLEY (1878). It may also be due to the increased flow of atropine-free blood through the gland brought about by stimulation of the chorda; even without stimulation the effect is transient. As soon as chorda stimulation begins to produce secretion, the flow of saliva may contribute to the removal of the atropine.

Nicotine injected into the duct was found to cause a secretion of saliva. Since the effect was abolished not only by atropine, but also by an intravenous dose of hexamethonium, nicotine seems to exert its secretory effect on the parasympathetic ganglion cells. The maximal rate of secretion which could be obtained with nicotine was not very high, and much lower than with chorda stimulation, indicating that only some of the ganglion cells were reached by the nicotine, probably only those situated within the gland and not those at the hilus. The fact that hexamethonium injected into the duct in a dose which had no general effects could only to a small extent reduce the secretory action of chorda

stimulation speaks in favour of the view that only a small number of ganglion cells can be affected by drugs given into the duct.

The secretory effect of nicotine was found to be greater after section of the chorda. This may in part be due to a supersensitivity to acetylcholine, liberated at the postganglionic endings, and partly to an increased sensitivity of the decentralized ganglion cells to nicotine (EMMELIN 1953).

An important precaution in experiments of this type is, not to inject big volumes of fluid, since the ability of the gland cells to secrete in response to stimulating agents will otherwise get lost. HEIDENHAIN (1874) denounced this method of administration; his unfavourable experiences were, very likely, due to the fact that too big volumes of fluid had been given. BOTTAZZI et al. (1908) found that chorda stimulation was rendered ineffective by injection of adrenaline into the duct. This might possibly in part be due to a vasoconstriction caused by the drug, as these authors assumed. But probably it was mainly an unspecific effect of the very big volumes of fluid used in these experiments.

The experiments have failed to explain the mechanism by which secretion and vasodilatation is brought about after the injection of saline solution into the duct. It should be mentioned that secretory and vascular changes in the submaxillary gland of dogs after occlusion of the salivary duct or injection of saline or gum-saline have been described already by GESELL (1920).

Summary.

Injections of drugs into the submaxillary duct of cats (and in some cases dogs) have been tried as a way of affecting the activity of the gland. The drugs which are ordinarily used to cause a flow of saliva or to affect a flow already going on were tested. The effects typical to the drugs when given by vein were obtained. Both secretory and vascular responses to the drugs were found. Acetylcholine and pilocarpine caused secretion and vasodilation. Eserine enhanced the secretory effects of acetylcholine and chorda stimulation and caused a flow of saliva if given in bigger dose. Atropine antagonized these effects. Adrenaline gave secretion, counteracted by dihydroergotamine. Experiments with nicotine and hexamethonium indicated that some of the parasympathetic ganglion cells can be reached from the duct. Cocaine was found to

increase the secretory effect of noradrenaline, whereas the secretory and vascular effects of chorda or sympathetic stimulation were reduced or abolished and those of acetylcholine not affected except by large doses of cocaine.

This method of administration offers the following advantages. Very small doses of the drugs are required. With small or moderate doses the effects remain local in the gland; effects on distant organs do therefore not interfere with the experiment, and the contralateral gland can serve as a control. Drugs can be given in doses to produce concentrations within the gland which could in some instances not be reached were they injected intravenously. Drugs which given by vein have a long duration of action are found to act for a short time only, when administered by way of the duct, and the effects of repeated doses can therefore be compared.

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Effect of Adrenaline on the Interaction between Plasma and Tissue Constituents.

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FORSSBERG and one of us (1952) observed that the rate of interaction of labelled plasma phosphate with the phosphate of the liver of the mouse is markedly enhanced if the animals were previously exposed to a massive dose of X-rays. In the effect of irradiation with X-rays on the interchange between plasma and tissue constituents hormonal effects may be involved. This line of thought induced us to study the effect of adrenaline on the rate of interaction of plasma and tissue constituents. A large number of mice were injected intraperitoneally with labelled phosphate, partly after previous administration of adrenaline, partly without, and 15 min. later the distribution of P^{32} between liver, muscles, skeleton, and blood plasma was determined. The P^{32} content of the plasma was found to be markedly depressed in the adrenaline injected mice. As beside the rate of extrusion of the labelled phosphate from the circulating plasma its rate of resorption into the circulation may be influenced as well, we injected a tracer amount of labelled phosphate into the circulation of the rabbit with the aim to eliminate the last mentioned effect and followed its rate of disappearance. Adrenaline was found to increase very markedly the rate of extrusion of P^{32} from the vascular bed also in this case.

The rate at which phosphate passes the capillary wall is a very high one. In order to investigate the effect of adrenaline on a slowly penetrating compound we studied the rate of extrusion

of intravenously as iron-β-globulin injected labelled iron in the controls and in the adrenaline injected rabbits. Administration of adrenaline was found to accelerate the exodus of the slowly disappearing Fe^{59} from the circulation. We determined furthermore the rate of exodus of radiosodium from the circulation.

Experimental.

In each of our first thirteen experiments, 10 controls and 10 adrenaline administered mice of about 18 grams were injected with 0.1 ml saline containing 0.3 microcurie of P^{32} present as carrier-free sodium phosphate. 15 min. later, the mice were decapitated and the P^{32} content of dry plasma, liver, femur, and gastrocnemius tissue of the same weight of controls and adrenaline administered mice was compared. 2 to 20 micrograms of adrenaline chloride dissolved in 0.1 ml saline were injected subcutaneously to every second mouse 10—40 min. before administration of P^{32}. The controls were injected at a corresponding time with 0.1 ml saline which did not contain adrenaline. In the later series of ten experiments, 10 micrograms of adrenaline dissolved in 0.1 ml of saline were injected subcutaneously to every second mouse 20 min. before the administration of P^{32}. After the lapse of further 15 min. the mice were decapitated. The adrenaline preparation (exadrin) used in our experiments was a generous gift of Astra.

In the experiment in which the rate of disappearance of the intravenously injected P^{32} was followed, rabbits weighing 2—3 kg and previously injected with urethane (1.5 g per kg body weight) were injected into the vena jugularis with 0.1 ml of saline containing 0.1 mg of P^{31} and P^{32} of 25 μC activity. Plasma samples of about 0.5 ml were secured at intervals from the carotid. The technique used was thus the same as applied by HAHN and one of us (1941), by FLEXNER and his colleagues (1942), and numerous other experimentors. Another group of sister rabbits was injected subcutaneously 24 min. before the start of the experiment with 40 micrograms of adrenaline.

In our investigations on the effect of adrenaline on Fe^{59} extrusion, we injected labelled $FeCl_3$ containing about 3 micrograms of iron and 1 microcurie of activity into the ear vein of a rabbit and, $1^1/_4$ hour later, when its plasma contained the Fe^{59} almost exclusively as β-globulin, 6 ml of the plasma of this rabbit were transfused to a sister rabbit. Pharmacological doses of adrenaline were administered by subcutaneous, physiological doses (1 γ per minute per kg body weight) by intravenous infusion all through the experiment and for the last 10 min. before its beginning.

About 0.5 ml of plasma was secured from the carotid at intervals also in these experiments. After wet ashing of the plasma sample 500 micrograms of iron were added, the iron precipitated as FeS, as described by AGNER, BONNICHSEN and one of us (1954), filtered through a perforated aluminium dish, and its activity measured.

In five experiments with mice 0.2 γ of labelled $FeCl_3$ was injected intraperitoneally to each of 20—50 mice, half of which were injected with 1 to 10 γ of adrenaline. The mice were killed 50 min. after being injected and the Fe^{59} content of the plasma and organs was determined.

Results.

a) Effect of adrenaline on the distribution of intraperitoneally injected P^{32} in the mouse.

The result of the preliminary experiment carried out with 220 mice is an absence of a significant difference between the P^{32} content of the dry liver samples and dry muscle samples of the same weight of adrenaline injected mice and of controls, the ratio being 0.99 and 0.95. Administration of adrenaline, however, led to a by 20 p.c. depressed uptake of P^{32} by the dry femur. The plasma of the adrenaline injected mice contained at the end of the experiment which took 15 min. 29 p.c. P^{32} only of that of the controls.

In the final experiments, administration of adrenaline as seen in Table 1 did not significantly influence the uptake by the liver (ratio 0.97) and only slightly that by the muscles (ratio 0.94), but it strongly depressed, by 40 p.c., the uptake by the femur and also the P^{32} content of the plasma, the mean value of which was reduced by 32 p.c., as seen in Table 1.

It may be due to a decreased circulation rate in the bone tissue that, under the action of adrenaline, incorporation of P^{32} into the skeleton is reduced. Furthermore recrystallization of the bone apatite which to a large extent is responsible for the P^{32} incorporation is presumably an enzymatic process and it is quite possible that adrenaline interferes with the latter.

The markedly lower P^{32} content of the plasma of adrenaline injected mice could be due to a lower resorption rate due to the effect of adrenaline or an increased rate of efflux from the circulation. It is hardly probable that the former is the case. Adrenaline was found (1954) to decrease the resorption rate of intraperitoneally injected bicarbonate, the difference in the rate of resorption due to the presence of adrenaline manifests itself however in the course of the first few minutes only. We can expect phosphate to show a similar behaviour. To make sure that we are confronted with an enhanced rate of loss of P^{32} by the plasma under the effect of adrenaline we injected labelled phosphate intravenously into rabbits, eliminating thus the possible rôle of a resorption process.

Table 1.

Ratio of the P^{32} content of the same dry weight of organs of adrenaline injected and of control mice 15 min. after intraperitoneal injection.

Ratio of P^{32} content	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8	Exp. 9	Exp. 10	Mean value
$\frac{\text{Plasma}}{\text{Inj. } P^{32}}$ contr. $\times 10^{-2}$	3.48	1.54	1.905	2.08	1.03	1.81	1.595	1.27	1.58	1.0	1.729
$\frac{\text{Plasma}}{\text{Inj. } P^{32}}$ adren. $\times 10^{-2}$	2.85	1.08	1.51	1.342	0.728	1.06	1.09	0.775	0.787	0.735	1.196
$\frac{\text{Plasma}}{\text{Inj. } P^{32}}$ adr.: $\frac{\text{Plasma}}{\text{Inj. } P^{32}}$ contr.	0.82	0.701	0.794	0.645	0.71	0.585	0.685	0.61	0.498	0.735	0.678
Of plasma and liver in controls	0.897	0.517	0.696	1.01	0.883	0.745	0.855	0.562	0.801	0.591	0.756
Of plasma and bone in controls	2.2	1.931	2.19	2.78	2.0	2.28	2.17	1.871	2.21	2.35	2.198
Of plasma and muscle in controls	3.47	2.599	3.85	3.86	3.785	3.41	3.46	2.65	3.57	3.16	3.381
Of plasma and liver in adrenaline injected	0.65	0.308	0.512	0.634	0.733	0.528	0.614	0.298	0.487	0.502	0.527
Of plasma and bone in adrenaline injected	2.48	2.24	2.95	2.48	2.76	2.43	2.92	1.73	2.35	2.735	2.507
Of plasma and muscle in adrenaline injected	2.73	1.615	2.73	2.70	3.13	2.2	2.46	1.588	2.29	2.6	2.404
$\frac{\text{Plasma}}{\text{Liver}}$ adr.: $\frac{\text{Plasma}}{\text{Liver}}$ contr.	0.725	0.596	0.736	0.628	0.83	0.710	0.718	0.53	0.608	0.850	0.693
$\frac{\text{Plasma}}{\text{Bone}}$ adr.: $\frac{\text{Plasma}}{\text{Bone}}$ contr.	1.128	1.16	1.349	0.893	1.38	1.067	1.345	0.925	1.062	1.162	1.147
$\frac{\text{Plasma}}{\text{Muscle}}$ adr.: $\frac{\text{Plasma}}{\text{Muscle}}$ contr.	0.787	0.622	0.709	0.700	0.827	0.645	0.712	0.598	0.642	0.824	0.707
$\frac{\text{Liver}}{\text{Bone}}$ adr.: $\frac{\text{Liver}}{\text{Bone}}$ contr.	1.552	1.941	1.833	1.421	1.665	1.503	1.876	1.794	1.741	1.368	1.665
$\frac{\text{Liver}}{\text{Muscle}}$ adr.: $\frac{\text{Liver}}{\text{Muscle}}$ contr.	1.081	1.049	0.696	1.115	1.0	0.909	0.99	1.128	1.05	0.967	1.026

The decrease observed in the P^{32} content of the plasma of the adrenaline injected mouse is due to an increased rate of interchange between plasma and extravascular phosphate and not to an increased exodus of plasma phosphate. This follows from the fact that, in our experiments with mice taking 15 min., the inorganic P content of the plasma is not influenced by the presence of adrenaline, as seen in Table 2.

Table 2.

Inorganic P content of the plasma of controls and of adrenaline injected mice.

Groups of 10 mice each	Inorganic P mg%	
	Controls	Adrenaline injected
1.....	5.40	5.27
2.....	5.08	5.03
3.....	4.64	6.34
4.....	7.10	6.38
5.....	6.48	—
6.....	5.88	6.23
7.....	6.38	5.50
8.....	5.52	6.41
9.....	4.70	4.05
10.....	9.27	8.54
11.....	6.73	5.85
12.....	5.87	6.50
Mean value	6.09±1.28	6.12±1.14

Pincus and assoc. (1933) investigated the effect of subcutaneously administered 80 micrograms/kg of adrenaline on the inorganic phosphate content of rabbit plasma. After the lapse of 15 min., they found 1 p.c. decrease only, one of 12 p.c. after the lapse of 1 hr. The observation that the inorganic P content of the plasma is reduced several hours after administration of adrenaline was reported by Cori (1930) at an early date.

Effect of adrenaline on the rate of extrusion of intravenously injected labelled phosphate from the circulation of the rabbit.

Fig. 1 demonstrates the rate of disappearance of the intravenously injected P^{32}, 0.2 ml saline containing 0.1 mg P as labelled phosphate, from the circulation both in a control rabbit and in a sister rabbit to which 20 γ of adrenaline were administered subcutaneously 17 min. before and again 26 min. after P^{32} was injected.

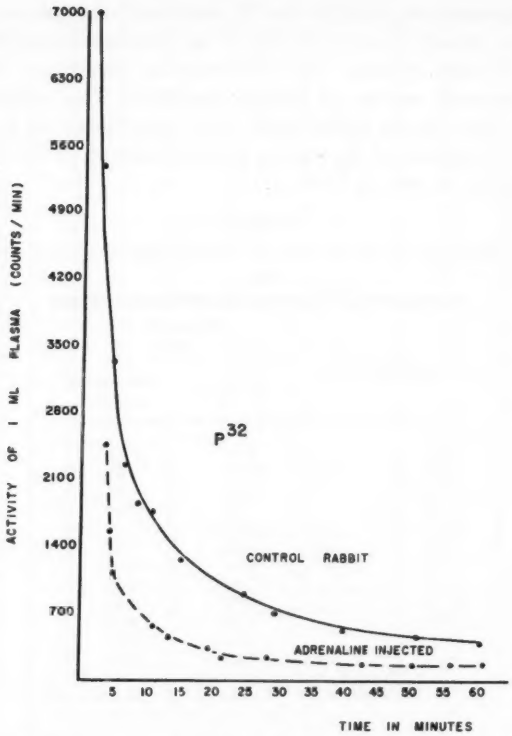

Fig. 1. Effect of subcutaneous injection of adrenaline on the rate of extrusion of P^{32} from the circulation of the rabbit.

Several experiments yielding similar results were carried out. The rate of disappearance of P^{32} from the circulation is markedly accelerated in all cases investigated. The phosphate which left the circulation may remain in the interspaces into which it first penetrates, it may return into the circulation or penetrate into the tissue cells, the last mentioned process becoming more and more predominant with increasing time. The effect of adrenaline is most pronounced in the early phase of the experiment.

Effect of adrenoxyl on the rate of phosphate interchange between plasma and tissue.

Adrenoxyl, the monosemicarbazone of adrenochrome, is endowed with a marked haemostatic action. It reduces the mean

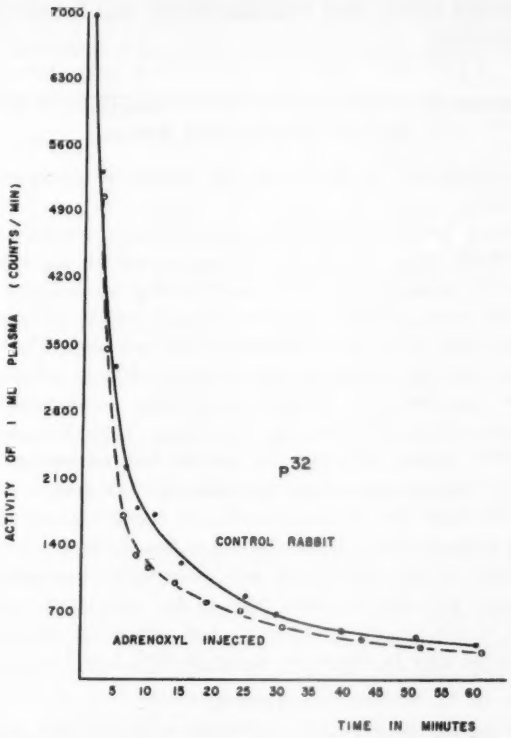

Fig. 2. Effect of subcutaneous and intravenous injection of adrenoxyl on the rate of extrusion of P^{32} from the circulation of the rabbit.

bleeding time appreciably. We wished to investigate whether the rate of capillary passage can be influence by administration of adrenoxyl. While 1 mg of adrenoxyl administered to humans was found to reduce the bleeding time by 30 p.c. — Roskam (1947) — 0.41 mg of the drug given by subcutaneous injection 1 to 3 hours before injecting the P^{32}, and the same amount injected again intravenously to the rabbit shortly before the administration of P^{32}, did not diminish the rate of passage of labelled phosphate through the capillary wall, as seen in Fig. 2, which indicates even a somewhat increased rate of passage.

The adenochrome Labaz was kindly presented to us by the Company Labaz and by Kabi A.B. and, in an investigation most

kindly carried out by Prof. ULF VON EULER, was found to be free from adrenaline.

Effect of Adrenaline on the Interaction of Fe^{59} between Plasma and Tissue.

a) *Disappearance of Fe^{59} from the plasma of intraperitoneally injected mice.*

We tested the rate of disappearance of intraperitoneally injected labelled $FeCl_3$ from the plasma of the mouse. 10 mg saline containing 0.04 micrograms of Fe, and having an activity of 0.05 microcurie, were injected both to controls and to 20 min. earlier subcutaneously with 10 γ adrenaline injected mice. The activity of the same volume of plasma was compared 50 min. after injecting the Fe^{59}. As seen from Table 3, adrenaline accelerates the disappearance of the Fe^{59} from the circulation. Table 4 contains data on the Fe^{59} content of organs of controls and adrenaline injected mice. The liver of adrenaline injected mice takes up 1.9 times as much Fe^{59} than that of the controls. In another group of experiments a corresponding ratio of 1.8 was found. As 1 ml of plasma at the end of the experiment had an activity corresponding to 860 counts per min. a minor part of the difference in the Fe^{59} content of the organs of controls and adrenaline injected mice (620 counts) may be due to a change in their blood content.

Table 3.

Ratio of the Fe^{59} content of 1 g of pooled plasma of 10 controls and 10 adrenaline injected mice.

Number of experiment	Ratio of activity control : adrenaline
1.....	1.72
2.....	2.40
3.....	1.37

b) *Disappearance of Fe^{59} from the circulation of rabbits into which labelled iron-β-globuline containing plasma was injected.*

FLEXNER and assoc. (1948) have shown that after transfusing plasma, to which labelled $FeCl_3$ was added, in vitro to a guinea pig, the activity of the plasma decreases first with a half-time of about 20 min. but, after the lapse of 20—30 min., a further decrease in the plasma activity takes place with a half-value of

Table 4.

Effect of adrenaline on the uptake of intraperitoneally injected Fe^{59} by the pooled organs of 10 mice weighing 166 (C) and 176 (A) g.

Organ	Fresh weight in g	Dry weight in g	Counts/min. given by total tissue	Percentage change due to adrenaline
Liver C	6.8907	1.9105	4,560	+ 90.7
Liver A	7.5583	2.1985	8,690	
Spleen C	0.8765	0.2106	1,685	— 49.9
Spleen A	0.5586	0.1361	845	
Lungs C	1.2635	0.2831	711	+ 21
Lungs A	1.7735	0.3754	860	
Kidneys C	1.5907	0.4243	514	+ 52
Kidneys A	2.0982	0.5868	795	
Muscles C	2.2586	0.5704	800	— 61.7
Muscles A	2.9593	0.7494	306	

C denotes Controls. — A denotes adrenaline injected mice.

about 3 hrs. The initial rapid disappearance of plasma activity is due to the exodus of Fe^{59} which had no opportunity to combine with β_1-globulin, while the remaining iron which had opportunity to combine with that protein escapes at a much reduced rate.

In a preliminary experiment we injected into the ear vein of a donor rabbit 84 γ of labelled iron and transfused 18 ml of whole blood after the lapse of 1 hour to a sister rabbit. This procedure proved not to be satisfactory, half of the transfused Fe^{59} disappearing from the circulation already in the course of 27 min., a further half in the course of 60 min. We then injected the donor rabbit with 1 γ of iron only having an activity of 0.9 micro C, and after the lapse of 1 hr. transfused to a sister rabbit 10 ml of the plasma of the donor, securing at intervals plasma samples from the receptor. The result of this and a second similar experiment is seen in Fig. 5.

The experiment was then repeated with receptor rabbits to each of which 20 γ of adrenaline were administered subcutaneously 52 min. and 33 min. before transfusing the activity plasma.

In another experiment we administered a physiological dose of adrenaline to the recipient rabbit, infusing all through the experiment 0.5 ml of saline per min. containing 1 γ of adrenaline per kg body weight.

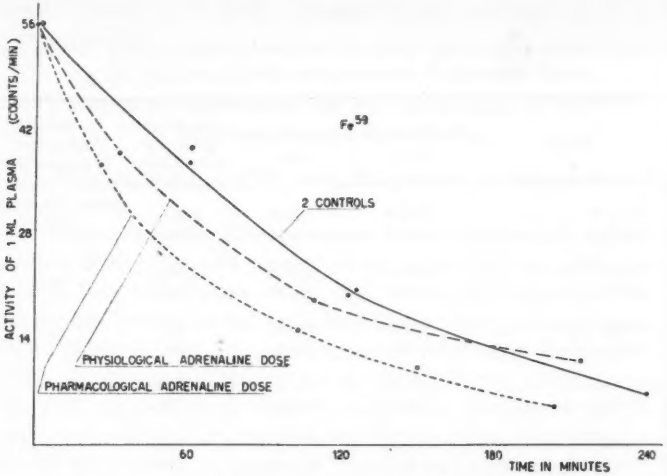

Fig. 3. Effect of injection of adrenaline on the rate of extrusion of Fe^{59} from the plasma of the rabbit.

In both experiments a marked increase of the disappearance of Fe^{59} from the circulation was observed under the action of adrenaline, the effect of a pharmacological dose being the more pronounced one.

Discussion.

Following injection of labelled ions into the jugular vein there is a large arteriovenous concentration difference which decreases exponentially with time (PAPPENHEIMER 1950; SCHLOERB 1950). It is conceivable that adrenaline accelerates this decrease and thus accelerates the extrusion of the labelled ions from the vascular bed. The rate of extrusion may be determined by the rate of blood flow which is accelerated by small doses of adrenaline. The fact that adrenaline influences markedly the rate of passage of intravenously injected P^{32} as phosphate or Fe^{59} circulating as β-globulin into the extravascular space is not necessarily to be interpreted as due to a change produced in the permeability of the capillary wall. Phosphate which passes from the vascular bed into the interspaces may repeatedly return into the former and escape again. If, however, it took its way from the interspaces into the tissue cells, the chance of returning is a rather small one in view

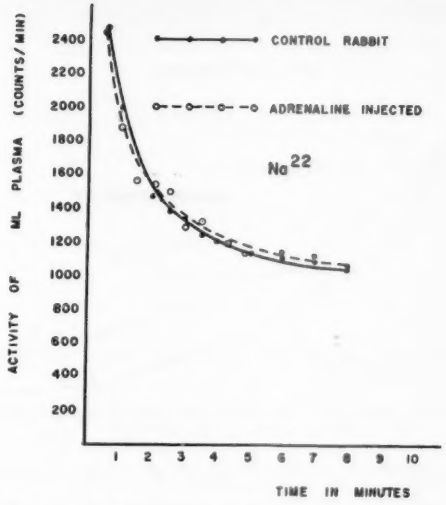

Fig. 4. Effect of injection of adrenaline on the rate of extrusion of Na^{22} from the circulation of the rabbit.

of the large phosphate pool of tissue cells. An escape of the P^{32} into the cells will thus reduce the probability of its return from the interspaces into the vascular bed and hence will ultimately accelerate the rate of exodus of P^{32} from the plasma.

That injected K^{42} leaves the plasma at a much more rapid rate than injected Na^{24} was observed in the earliest experiment of this type (HAHN 1941) and interpreted as due to the large potassium pool of the tissue cells in contrast to their restricted sodium pool. Should adrenaline accelerate the rate of interchange between the phosphate of the interspaces and that of the tissue cells, this may explain its effect on the rate of exodus of P^{32} from the vascular bed. Doses of adrenaline as applied by us are known to increase the oxygen consumption (CARR 1934, LUNDHOLM 1949).

In the case of sodium the interchange between interspaces and tissue cells having a restricted importance only, as the incorporation of Na^{24} into the skeleton and cartilage which harbours most of the not extracellular sodium takes some time, adrenaline should not much accelerate the exodus of Na^{24} in experiments taking about 1 hour only.

It is of interest to note that while from the sodium content of

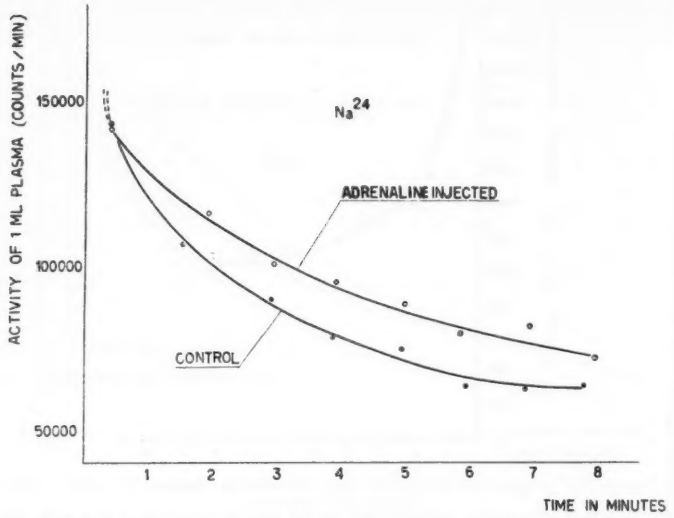

Fig. 5. Effect of injection of adrenaline on the rate of extrusion of Na^{24} from the circulation of the rabbit.

the ear cartilage of the rabbit follows an apparent extracellular volume of that organ amounting to 96.6 p.c. of its weight, we found 10 min. following the injection of radiosodium into the circulation of the rabbit a Na^{22} content of the ear cartilage which corresponds to an extracellular space of 5 p.c. only. Thus interchange between plasma sodium and the extracellular sodium of the ear cartilage is a slow one. Injection of adrenaline increased the last mentioned figure to 10 %.

We could not observe any acceleration of the exodus of Na^{24} from the circulation under the action of adrenaline (cf. Fig. 4), in some of our experiments (see Fig. 5) even a decrease in the rate of extrusion of radiosodium took place. An observation which may be taken to support the interpretation of the accelerated disappearance of P^{32} and of Fe^{59} of the plasma under the effect of adrenaline as to be due to an enhanced interaction rate between the phosphate resp. iron present in the tissue cells and that present in the interspaces. DOBSON and his associates (1953) found that the removal rate of sodium injected into an artery is greatly accelerated by a systematic administration of adrenaline.

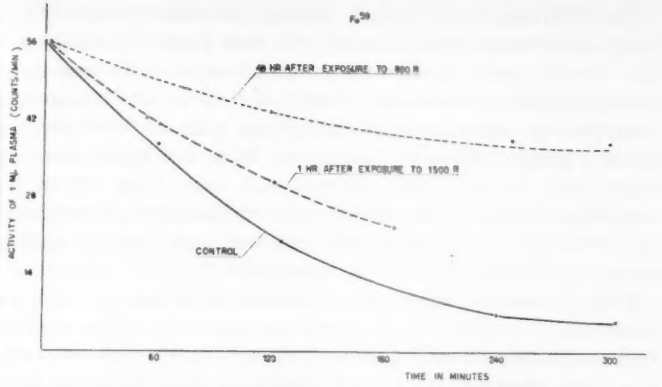

Fig. 6. Effect of irradiation with Roentgen rays on the rate of extrusion of Fe^{59} from the plasma of the rabbit.

The additional Fe^{59} given off by the plasma under the effect of adrenaline was found by us to find its way to a large extent into the liver in which presumably an enhanced iron turnover takes place under the effect of adrenaline. Adrenaline was observed to produce hypoferemia in dog by GUBLER (1950) and in human by BATEMAN (1952). Adrenaline was found to increase the oxygen consumption (CARR 1934; LUNDHOLM 1949).

From the activity of the injected plasma and of that of 1 ml plasma secured after the lapse of 3 min. follows a plasma content of the rabbit amounting to 80 ml. As the iron content of the plasma amounts to 1.8 γ per ml and the half-life of the plasma Fe^{59} to 1.5 hr. the amount of Fe^{59} turned over in the course of 1 hr. works out to be $\frac{0.693 \cdot 1.8 \cdot 80}{1.5} = 66\ \gamma$. Injection of a pharmacological dose of adrenaline increases thus the amount of plasma iron turned over per hour to 130 γ.

It is of interest to compare this increase with a decrease in the rate of plasma turnover due to total irradiation of the rabbit. As seen in Fig. 6 measured 1 hr. after irradiation with a high dose of 1,500 r the amount of plasma iron turned over in the course of 1 hr. decreased to 42 γ. Measured 48 hr. after irradiation with 800 r only the decrease is much more pronounced the amount turned over being less than 30 γ.

The difference in the rate of plasma iron turnover shortly and 2 days after exposure is presumably at least partly due to the fact that shortly after irradiation orthochromatic and even polychromatic erythroblasts are present in marrow which were not destroyed by exposure to radiation and take up Fe^{59} previous to their removal into the circulation. After the lapse of several hours these erythroblasts matured and were given off to the circulation but were not renewed in the marrow as irradiation suppresses the formation of new cells and even destroys such as are in an earlier phase of their maturation.

Experiments in which massive doses of adrenoxyl were administered to rabbits did not reveal any tightening of the capillary wall to the passage of P^{32}, a small increase in the rate of exodus was even observed. Adrenoxyl influences thus the interchange between the vascular and extravascular P^{32} in a similar sense, though to a much restricted extent, as does adrenaline.

Adrenaline is known to bring about a violent increase in the outflux of sodium through surviving frog skin, while to increase moderately only the influx of sodium (Ussing 1952). A third compartment in which sodium could accumulate was absent in these experiments.

Gemzell and Samuels (1950) investigated the effect of ACTH on the P^{32} content of the plasma of rats injected intraperitoneally with radiophosphate. They found the administration of the adrenocorticotrophic hormone to diminish with 15 p.c. both the P^{32} and P^{31} content of the plasma 50 min. after the injection of radiophosphate. No increase in the P^{32} content of the liver was observed. In hypophysectomized rats the P^{32} content of the plasma, however, is conserved for a longer time than in controls, as observed by Geschwind and assoc. (1950).

Summary.

Administration of adrenaline to mice before intraperitoneal injection of P^{32} leads to a markedly increased rate of passage of the resorbed P^{32} from the plasma into the tissues.

The rate at which P^{32} leaves the circulation of the rabbit after intravenous injection is accelerated to twice its normal value if a pharmacological dose of adrenal was administered previously.

Massive doses of adrenoxyl lead to a slightly increased rate of exodus of the intravenously injected P^{32} from the circulation of the rabbit.

After intraperitoneal injection of $Fe^{59}Cl_3$ to mice Fe^{59} leaves at a much enhanced rate the plasma after administration of adrenaline. Much of the Fe^{59} is recovered in the liver.

Labelled iron of Fe^{59}-β_1-globulin transfused with the plasma of a donor rabbit to a recipient rabbit leaves the circulation if a pharmacological dose of adrenaline is administered at a twice accelerated rate.

In contrast to administration of adrenaline exposure to X-rays strongly decreases the exodus of Fe^{59}.

Adrenaline does not accelerate the rate of exodus of intravenously injected labelled sodium from the circulation of the rabbit.

The senior author's thanks are due to the Swedish State Research Council for generous support of this investigation.

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Kinetics of Galactose Elimination.

By

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Received 30 June 1954.

Galactose is known to be a substance well suited for the estimation of the liver function (BAUER 1906, DRAUDT 1913, SHAY, SCHLOSS and BELL 1931, BOLLMAN, MANN and POWER 1935). To get the best quantitative measure of its metabolism, it is of primary importance to know the rate at which it is removed from the blood.

The galactose blood-time curve has by FISHBERG (1930), DOMINGUEZ and POMERENE (1944) and STENSTAM (1946) been described as a decreasing exponential function which means that the amount of galactose removed from the blood is dependent on the concentration. The equation of the simple decreasing exponential function is

$$c = c_0 \cdot e^{-g \cdot t} \qquad (1)$$

(c is the concentration, t time, e the base of natural logarithms and c_0 and g are constants).

Preliminary experiments with intravenous administration of galactose have suggested to us that the amount of galactose eliminated per time unit is constant, independent of concentration within a certain range. Determination of the difference of galactose concentration in peripheral blood and blood obtained by catheterization of the hepatic veins has shown this difference to be constant above a certain level in the peripheral blood. When

[1] Aided by grants from "Danish State Research Foundation" and "Miss P. A. Brandt's Legacy".

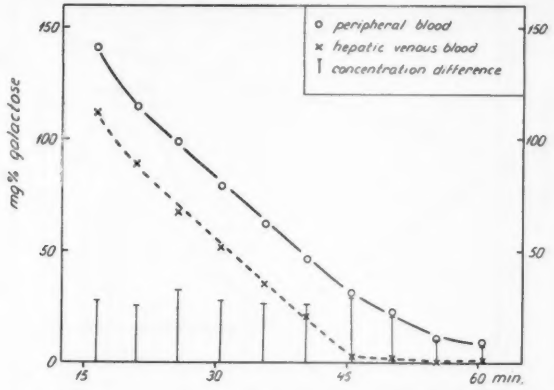

Fig. 1. *Catheterization experiment.* O. K. male, 17 years. 24 g of galactose intravenously in 5 minutes. Average concentration difference until about 45 minutes: 28 mg per cent.

this level is reached the galactose content of the hepatic veins approximates zero. A curve is shown in fig. 1. The catheterization experiments will be reported in detail in a separate communication (TYBJÆRG HANSEN, WINKLER and TYGSTRUP, to be published).

Furthermore, experiments with constant infusion of galactose (after a priming dose to shorten the time of equilibration and to raise the concentration to about 100 mg per cent) show that a constant plasma level only is found if the concentration falls below 50 to 30 mg per cent. Similar conclusions can be drawn from STENSTAM's results (1946). Fig. 2 shows an experiment in which 0.51 g of galactose was infused per minute after a priming dose of about 20 g, resulting in a continuous rise in concentration. The experiment was repeated a few days later in the same subject, only reducing the amount infused by ab. 50 per cent, which caused the concentration to fall until a level was obtained at ab. 30 mg per cent, as seen in the same figure.

The object of the present work is to decide whether the constant elimination, seen in these experiments, determines the shape of the elimination curve after a single intravenous injection which is the method generally employed in clinical intravenous galactose tolerance tests. If this be the case, expressions derived from equation (1), as for instance the "Galactose Removal Constant" of

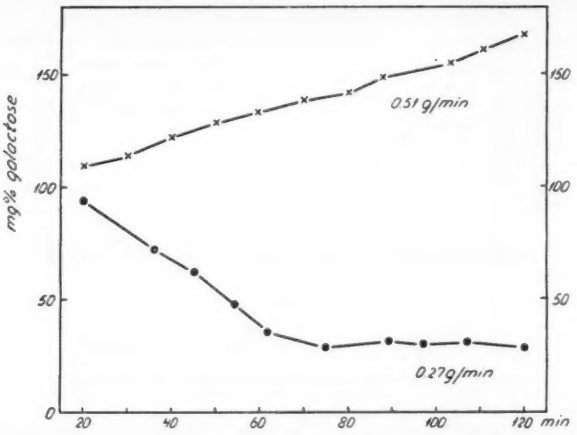

Fig. 2. *Infusion experiment.* K. S. male, 25 years. Priming dose 20 g of galactose, infusion of 0.51 g/min. (upper curve), and 0.27 g/min. (lower curve).

COLCHER, PATEK and KENDALL (1946) cannot be expected to describe the galactose elimination.

Therefore we have investigated the elimination curve after a single injection using frequent blood sampling in order to determine its course more accurately.

Methods.

Sixteen galactose tolerance tests have been carried out in 12 male subjects of 15 to 57 years of age, with no history of liver disease, and with normal liver function tests. The test was done with the subject in the recumbent position, at least 3 hours after a meal. 0.5 g per kg body weight of galactose (Sandoz, puriss.) dissolved in 100 ml of sterile water was injected intravenously at a constant rate of 20 ml per minute. Before the injection and at intervals of 3 to 5 minutes during the first hour or so afterwards, venous blood was drawn through an indwelling needle (Gordh-needle) into centrifuge tubes, containing one drop of a 5 per cent heparin solution and 4 mg of potassium fluoride.

The galactose concentration of plasma was determined spectrophotometrically in protein-free filtrates after oxidation of glucose to nonreducing gluconic acid by a specific glucose oxidase Notatin[1]. The procedure is described in detail elsewhere (TYGSTRUP, WINKLER, LUND and ENGELL 1954). Its standard deviation is approximately 2 mg per cent.

[1] Kindly supplied by Boot Pure Drugs Ltd. Nottingham, Engl.

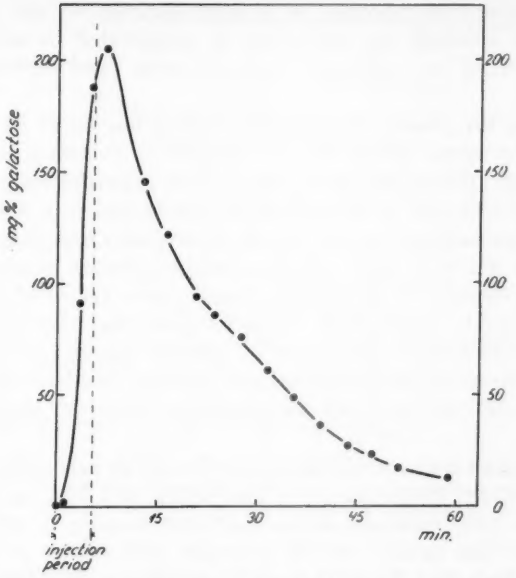

Fig. 3. *Single injection experiment.* W. B. male, 20 years. 32 g of galactose intravenously.

General Considerations.

A survey of the curves, obtained by plotting the plasma concentration against time as shown in fig. 3, reveals that each curve roughly can be divided into three sections, the first part consisting of a steep rise in concentration during the injection, with a maximum of 200 to 300 mg per cent about one minute after conclusion of the administration, followed by a rapid decrease in concentration for the next 10 to 15 minutes. During this period galactose removal from the blood is largely governed by diffusion to extravascular beds and by renal excretion (v. i.), therefore the curve is too complicated for study of the metabolism of galactose ("utilization"). This part of the curve has been examined in only 5 cases, while its transition into the second part has been ascertained in all cases.

During the second part the curves assume a more gradual decline for some time, averaging 22 minutes (12 to 29 minutes).

This interval is thought of greatest interest to the problem studied, although the curve may be complicated by incomplete equilibration of galactose between intra- and extravascular beds.

When the plasma concentration falls below 50 to 30 mg per cent, the curves flatten out, the elimination evidently undergoes a change (DOMINGUEZ et al. 1944). This concentration seems to correspond to the level at which the concentration in the hepatic veins approximates zero in the catheterization experiments, and also to the level below which a constant plasma concentration can be obtained in the infusion experiments. Traces of galactose often may be found in the blood for more than two hours after the administration. The more complicated kinetics of this part of the curves are not considered here because the relatively greater analytical error at small concentrations tends to obscure the details.

Therefore we have examined only the middle part of the curves. In each case it was delimited graphically, and this was possible because the transition of one part into another is sufficiently abrupt. The section studied generally will consist of samples drawn later than 20 minutes after beginning of the injection (the time required for distribution to extravascular beds?) and containing more than about 40 mg per cent (corresponding to the level of changed elimination).

Results.

If the amount of galactose removed from the blood after a single intravenous injection is independent of the concentration in the interval studied, the blood galactose curve here would be a straight line, the equation of which is

$$c = c_0 - g \cdot t \qquad (2)$$

The fitting of the curves to equation (1) and (2) has been investigated in the following way. For each curve two regression equations were calculated by means of the method of least squares (*e. g.* FISHER 1950). In one calculation the concentrations and the other the logarithms of the concentrations were used as dependent variate, time being independent variate in both. The deviation of each experimentally found concentration from the value calculated from these two equations, in units of the standard

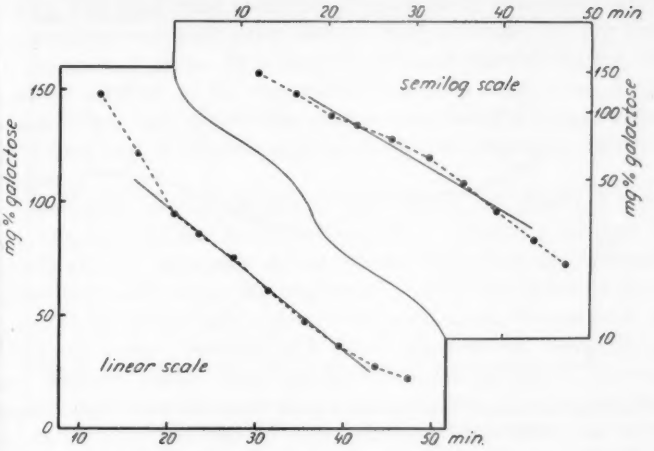

Fig. 4. Middle section of the galactose curve of fig. III in linear and semilogarithmic scale. ● - - - - ● experimentally found curve, ——— regression line. Linear equation: $c = 160.9 - 3.172 \cdot t$; $s = 1.0186$. Exponential equation: $\log c = 2.4616 - 0.0224 \cdot t$; $s = 0.00258$.

error of estimate (s) (HALD 1948, p. 406) has been determined ($\frac{c-C}{s}$ and $\frac{\log c - \log C}{s}$ respectively). A curve in linear and semilogarithmic scale with the corresponding regression lines is seen in fig. 4. Fig. 5 illustrates the deviations from the linear curve in all 16 experiments, and fig. 6 that from the exponential curve. In these figures the zero line represents the regression line used.

Discussion.

The criterion of fitting is random distribution of the deviations around the regression line. From figs. 5 and 6 it therefore appears that the curve-section studied is rectilinear while it deviates significantly from the exponential shape.

Furthermore the fitting of the galactose curves is the best obtainable with the analytical procedure employed, as the standard deviation of the analysis at the average concentration (80 mg per cent) is 1.8 mg per cent, corresponding to 0.0100 in the logarithmic system (HALD 1948, p. 193) and the average standard error of estimate of the linear curves is 2.0 mg per cent, while that of the exponential curves amounts to 0.0240.

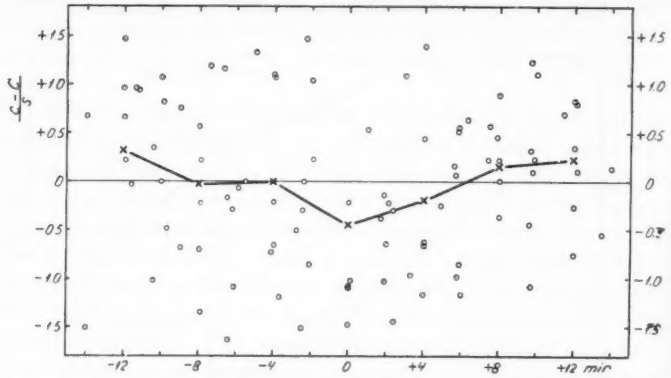

Fig. 5. Deviation from *linear* regression line of the chosen points in all 16 experiments (related to average time of the individual curve).

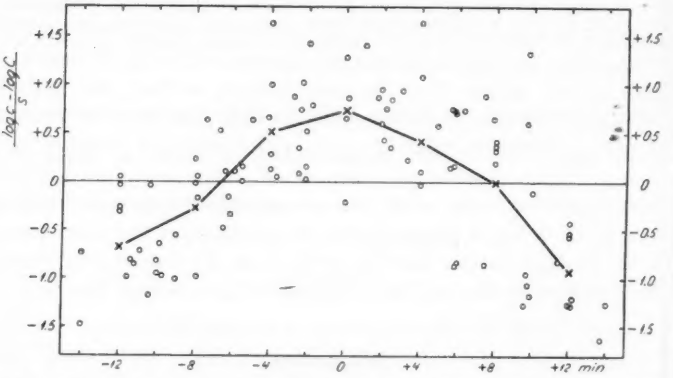

Fig. 6. Deviation from *exponential* regression line of the chosen points in all 16 experiments.

Whether there is some preponderance of negative values of the deviations in fig. 5 around the middle part of the line, balanced by positive ones at the extremities, indicating a slight exponential component, cannot be decided with certainty, because the deviation, if there be any, is of the same order of magnitude as the analytical error.

An exponential component might be attributed to renal excretion, which is known to be proportional to concentration (GAMMELTOFT and KJERULF-JENSEN 1943. DOMINGUEZ et al.

1944). The total renal excretion of galactose in our experiments, determined polarimetrically (GEILL 1934), averages 8.8 per cent of the amount given. In 5 cases the amount excreted during the first 20 minutes of the experiment has been determined to be about 80 per cent of the total amount excreted which means that only 2 per cent of the amount given is excreted during the interval studied.

Calculation of the volume of distribution of galactose from the quantity utilized (amount injected — amount recovered in urine) and the arbitrary initial plasma concentration, obtained by extrapolation from the rectilinear part of the curve to zero-time, yields an average value of 14 l, or about 20 per cent of the body weight, (extracellular fluid? (DOMINGUEZ 1950)). WICK and DRURY (1953) find that C-14 labelled galactose, injected with carrier in eviscerated rats, during the first hour is distributed in 20 to 25 per cent of body weight. If the exponential curve is used for extrapolation, the volume calculated averages 10 l. The value obtained by the first method seems independent of the slope of the rectilinear part of the curve, as it is the same in 6 patients with decreased galactose elimination as in the normals. We think this may support the concept of rectilinearity.

Conclusions.

These experiments indicate that a constant galactose elimination, such as is found in the catheterization and infusion experiments, results in a rectilinear part of the blood galactose curve after a single intravenous injection. It seems relevant to take this into consideration when estimating the liver function from intravenous galactose tolerance tests. Probably the slope of the curve is a measure of the galactose removal capacity of the liver (L_m of galactose (LEWIS 1952)).

Summary.

The galactose elimination curve in blood after a single intravenous injection is studied. Only a part of the curve is used for elimination analysis. This part is shown to be rectilinear which means that the amount of galactose removed is independent of concentration and therefore probably a measure of the galactose removal capacity of the liver.

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Investigations on the Plasma Phosphate.

II. Diffusibility of the Inorganic Phosphate of Guinea Pig Serum.

By

ANNA-RIITTA FUCHS and FRITZ FUCHS.

Received 30 June 1954.

A number of observations give support to the belief that the plasma contains a labile, non-diffusible phosphorus compound, which, by the usual chemical methods for phosphorus determination, is broken down to inorganic phosphate and determined as such. Various methods have been used: ultrafiltration, various forms of dialysis, ultracentrifugation, and excretion studies with radioactive phosphorus as a tracer element. All in vitro studies have the drawback that it is extremely difficult, if not impossible, to handle the blood in such a way that all the constituents of the circulating blood are kept in the same chemical and physical state. If the supposedly non-diffusible phosphate fraction is very labile it is no wonder that such changes of the blood as addition of coagulation inhibiting compounds, separation of plasma and corpuscles and alteration of carbon dioxide tension may cause it to break down. This might explain the disagreement between the results of various investigators. In vivo experiments do not have these difficulties but are more complicated to perform, and even they seem to give contradictory results. In a previous paper (FUCHS and FUCHS (1954)) we have reported in vivo studies on the

[1] These investigations were supported by grants from "Købmand i Odense Johann og Hanne Weimann, f. Seedorffs Legat" and P. Carl Petersens Fond.

renal excretion of radioactive phosphorus in the guinea pig with the technique described by GOVAERTS (1948). We shall here report dialysis experiments on guinea pig serum, designed to give additional information on the question of a non-diffusible phosphate fraction. This question is of great interest, since the existence of a non-diffusible phosphate compound, which chemically is determined as inorganic phosphate, invalidates most of the phosphate distribution studies in mammals which have been carried out with the aid of radioactive phosphorus.

Procedure.

A type of compensation dialysis was used. The serum was dialyzed against a Ringer's or Tyrode's solution containing phosphate in approximately the same concentration as the serum. A hydrostatic pressure of the same order as the colloid osmotic pressure was applied upon the serum. A mixture of 5 % CO_2 and 95 % N_2 was led through the apparatus in order to keep the pH constant, and the temperature was kept at 37° C.

The apparatus was built up in the following way: The semipermeable membrane, of DuPont cellophane, was tied like a small bag to a rubber stopper and inserted into a cylindrical tube, closed with a larger stopper. Through the latter were led four steel cannulas, of which two continued through the smaller stopper to the inside of the bag. 2—4 ml of serum was put into the bag and 6—8 ml of the salt solution into the tube around the bag. The gas mixture was led through a washing bottle to the bag, from there to a mercury lock and then back to the outside of the bag. Through the lock a pressure corresponding to 20—22 mm of mercury was applied upon the serum. The apparatus was rocked in a water bath at 37° C. with small glass beads stirring the serum and the dialysate constantly.

The blood was withdrawn from the carotid artery of adult guinea pigs anaesthetized with Nembutal (30 mg per kg) intraperitoneally. It was collected in chilled syringes and tubes under mineral oil. After immediate centrifugation the plasma was separated from the cells and allowed to clot, still under oil and chilled. The serum was then transferred to the bag. The Tyrode solution was made with approximately the same phosphate concentration as the serum (35—60 γ P per ml).

During the dialysis samples of serum and dialysate were withdrawn through the cannulas to find out when equilibrium was reached. At the end the dialysate was tested for protein, and only experiments in which no protein had leaked through the membrane were accepted. The phosphorus was determined according to the UMBREIT (1945) modification of the method described by FISKE and SUBBAROW (1925), in the serum after precipitation of the protein with chilled 10 % trichloracetic acid. The water content of serum and dialysate was determined by drying aliquots of each sample.

Results.

Table 1.

The amount of non-diffusible phosphate calculated on the basis of a Donnan coefficient of 0.980.

Exp. No.	Time	Sample	No. of dialyses	P γ/ml before	P γ/ml after	P γ/ml H_2O	Calculated equilibrium	Non-diff. P γ/ml H_2O	Non-diff. P %
103	6 h.	Serum	1	60.0	58.5	61.6	57.9	3.7	*6.3*
		Ringer		69.6	59.6	60.2	60.2		
111	6½ h.	Serum	4	49.0	54.1	57.6	53.2	4.4	*7.6*
		Tyrode		35.7	54.7	55.3	55.3		
112	6½ h.	Serum	2	53.9	47.5	50.5	47.7	2.8	*5.5*
		Tyrode		54.7	49.1	49.5	49.5		
113	6 h.	Serum	2	31.0	44.8	47.4	45.2	2.2	*4.6*
		Tyrode		56.7	46.5	46.9	46.9		
114 a	5 h.	Serum	3	35.0	49.8	52.6	45.5	7.1	*13.5*
		Tyrode		56.0	46.9	47.3	47.3		
114 b	8 h.	Serum	3	35.0	45.5	48.1	43.4	4.7	*9.8*
		Tyrode		56.0	44.6	45.1	45.1		
Average non-diffusible phosphate									*7.9*

The results are shown in table 1. Two or more simultaneous dialyses were carried out in all experiments except the first and only the average results have been shown. In all experiments the concentration of phosphate per gram of water at the end of the dialysis was higher in the serum than in the dialysate even without a correction for the Donnan effect and even if the phosphate concentration had been higher in the Tyrode solution at the start. The differences are admittedly small as compared with the accuracy of the method of phosphorus determination used. The average variation in double determinations has in our hands been of the order of one γ. However, since all six experiments show the same, the results must be considered significant.

By in vivo dialysis in dogs GREENE and POWER (1931) found the ratio of Cl^- in plasma to Cl^- in dialysate to be 0.980. This value has been used in the present experiment for the correction for the Donnan equilibrium. In the blood the ratio HPO_4^{--} to

$H_2PO_4^-$ is approximately 4:1. This ratio has been used for the calculation in the following equation:

$$\frac{HPO_4^{--}\text{ dial.}}{HPO_4^{--}\text{ serum}} = \left(\frac{H_2PO_4^-\text{ dial.}}{H_2PO_4^-\text{ serum}}\right)^2 = \left(\frac{Cl^-\text{ dial.}}{Cl^-\text{ serum}}\right)^2 = \left(\frac{1}{0.980}\right)^2$$

GREENBERG and GUNTHER (1929—30) found the same Cl^- ratio (0.980) as GREENE and POWER (1931), when plasma protein was 5.7 %, and obtained a coefficient of 0.975 with 7 % protein. VAN SLYKE, WU and MCLEAN (1923) found a still lower ratio. A lower ratio means a higher percentage of non-diffusible phosphate than the calculated 7.9 % of the present series.

The disagreement in the first two experiments between the total phosphate in the system before and after the dialysis was due to water vapor being carried through the apparatus with the gas.

Discussion.

The filterability of calcium and of orthophosphate in the plasma has been the subject of a number of investigations. It has been shown that when the calcium and phosphate concentrations are increased above certain limits a colloid calcium phosphate may be formed. However, both in the present and in other investigations on the filterability of the plasma phosphate, a non-diffusible phosphate fraction has been found even with low phosphate concentrations, indicating the existence of another labile, non-diffusible compound. The results of different investigators are not quite in agreement, due probably both to variations in technique and to variations from species to species. With regard to technique it has been shown for instance that changes in the pH on account of loss of CO_2 may cause variations in the ratios between dialysate and plasma of sodium, chloride (GREENE and POWER (1931)), calcium, and phosphate (HOPKINS, HOWARD and EISENBERG (1952)). Certain anticoagulants change the ratio for calcium (STEWART and PERCIVAL (1928) and others) and probably even for phosphate.

The vivodiffusion studies on dogs by GREENE and POWER (1931) are probably those which have been carried out under the most physiological conditions. They gave a ratio between dialysate and plasma of 1.17 for phosphate as compared to the ratio of 0.98 for the supposedly completely diffusible chloride, indicating a non-

diffusible phosphate fraction of 19 %. In ultrafiltration studies on dog serum the non-filterable fraction varies from 0—5 % (SCHMIDT and GREENBERG (1945)) up to 25 % (BENJAMIN and HESS (1933)). While GROLLMAN (1927) found non-diffusible phosphate only when the calcium and phosphate levels were raised, SMITH, OLLAYOS and WINKLER (1943) found 12.5 % non-diffusible phosphate both with normal and elevated phosphate concentrations. In immature rats BENJAMIN and HESS (1933) found up to 50 %, while neither GROLLMAN (1927) nor NEUHAUSEN and PINCUS (1923) found any non-diffusible phosphate in the pig.

In the human, ultrafiltration studies have given results varying from a few per cent (SMITH, OLLAYOS and WINKLER (1943)), 5—10 % (HOPKINS, HOWARD and EISENBERG (1952)) up to 20 % (BRAIN, KAY and MARSHALL (1928)). ELLIOT, HAHN and HEVESY (1948) electrodialyzed plasma, to which radioactive phosphate was added, and found 6—10 % non-diffusible phosphate.

The present studies seem to confirm the existence of a non-diffusible phosphate compound in the plasma of the guinea pig, but they do not give any information as to the nature of this compound.

Summary.

Compensation dialysis on guinea pig serum at 37° C. with constant carbon dioxide tension indicates the presence in the blood of a non-diffusible phosphate compound, which by the usual method for phosphorus determination is broken down and determined as inorganic phosphate. The average amount found in the present series was 8 % of the inorganic plasma phosphate.

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Investigations on the Plasma Phosphate.

III. Adsorption Studies on Guinea Pig Blood.

By

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Received 30 June 1954.

In two previous papers we have reported studies on the renal excretion of phosphate in the guinea pig with the aid of radioactive phosphorus, and on the diffusibility of the plasma phosphate in vitro by compensation dialysis (FUCHS and FUCHS 1954, 1954). Both series of experiments suggested the presence in the plasma of a non-diffusible phosphate compound, which by the usual methods for phosphorus determination is broken down and determined as inorganic phosphate. The amount of non-diffusible phosphate in the guinea pig plasma was found to be of the order of 8—10 % of the total inorganic phosphate.

Here we shall report adsorption experiments, in which guinea pig plasma or serum with added radioactive phosphate was shaken with precipitated barium sulphate in order to find out whether the radioactive phosphate and the original plasma phosphate was adsorbed to the same extent.

Procedure.

Blood was withdrawn from the carotid artery of adult guinea pigs under Nembutal anaesthesia (30 mg per kg intraperitoneally). Four of the animals were pregnant in the last week of gestation. From three of

These investigations were supported by grants from "Købmand i Odense Johann og Hanne Weimann, f. Seedorffs Legat" and P. Carl Petersens Fond.

these blood was also withdrawn from the umbilical cords. In one animal (No. 209) the blood was taken a couple of hours after a spontaneous delivery. In some experiments Heparin was used as anticoagulant, while in others the blood was centrifuged immediately and then allowed to clot.

Barium sulphate was prepared by precipitation from a solution of barium chloride or nitrate by addition of dilute sulphuric acid. The precipitate was freed from acid by washing, dried overnight at 100° C and stored in a desiccator.

To each sample of serum or plasma was added a minute amount of ^{32}P of a very high specific activity dissolved in saline. They were then vigorously shaken in well-stoppered glass tubes with barium sulphate in proportions varying from 5:1 to 5:3 for varying lengths of time in a shaking apparatus. All experiments were carried out at room temperature except No. 205, in which the samples were shaken at 8—10° C. After shaking the tubes were centrifuged, and aliquots of the supernatant were then taken for phosphorus determination and measurement of the radioactivity. The phosphorus determinations were carried out according to the UMBREIT (1945) modification of the method described by FISKE and SUBBAROW (1925).

Results and Discussion.

Table 1 shows the results with blood from non-pregnant guinea pigs and table 2 concerns blood from pregnant, puerperal and foetal guinea pigs. In all experiments where *serum* was used the specific activity of the serum inorganic phosphate was significantly lower after adsorption, *i. e.* a greater proportion of the added labelled ortophosphate was adsorbed than of the phosphate originally present in the serum. This would seem to indicate the presence in the serum of a phosphate fraction which behaves differently from the ortophosphate, but chemically is determined as such.

The amount of adsorbed phosphate increases with increasing amounts of barium sulphate, even above the 40 % (w/v) concentration which BENJAMIN and HESS have reported to give maximal adsorption. The specific activity of the residue is lowest after 15 minutes of shaking and increases with increasing shaking time.

Total acid-soluble phosphorus was determined before and after adsorption to find out whether the decreased specific activity could be due to the splitting of organic phosphate compounds during the experiment, but no changes were found.

With Ringer or Tyrode solutions the specific activity of the phosphate always remained unchanged. A slightly larger per-

Table 1.

Blood from non-pregnant guinea pigs. The last column shows the specific activity of the phosphate in solution after the adsorption in percentage of the original specific activity.

Exp. No.	Guinea pig	Sample	Amount $BaSO_4$ w/v %	Shaking time min.	P/ml. before	P/ml. after	Specific activity before	Specific activity after	Specific activity %
201	male	serum	40	120	40.8	9.1	21.90	16.36	*74.7*
202	male	serum	20	110	47.9	16.0	20.04	19.45	*97.0*
		—	40	110	47.9	10.7	20.04	17.70	*88.3*
203	male	serum	40	120	50.4	11.1	40.00	29.80	*73.3*
		—	60	120	50.4	12.4	40.00	20.60	*57.5*
		Ringer	40	120	50.1	10.4	27.96	27.25	*97.5*
		—	60	120	50.1	9.2	27.96	27.50	*98.4*
208	male	plasma	40	15	50.9	22.3	36.70	35.30	*96.1*
		—	—	52	50.9	19.0	36.70	37.80	*102.9*
211	male	plasma	40	14	38.3	19.3	81.90	83.54	*102.0*
		—	—	30	38.3	18.3	81.90	80.49	*98.2*
204	female	serum	60	15	56.3	17.0	162.7	80.6	*49.6*
		—	—	35	56.3	14.6	162.7	87.1	*53.5*
		—	—	130	56.3	10.0	162.7	130.8	*80.4*
		Tyrode	—	15	50.4	11.0	13.90	13.67	*98.4*
		—	—	35	50.4	11.0	13.90	13.71	*98.7*
		—	—	130	50.4	10.2	13.90	13.45	*96.7*

Table 2.

Blood from pregnant, puerperal (No. 209) and foetal guinea pigs.

Exp. No.	Sample	Amount $BaSO_4$ w/v %	Shaking time min.	P/ml before	P/ml after	Specific activity before	Specific activity after	Specific activity %
205	maternal plasma	40	15	46.7	17.7	1,760	1,728	*98.1*
	foetal plasma	—	15	87.5	41.5	718	714	*99.1*
	Tyrode	—	15	62.8	36.7	1,216	1,237	*101.7*
206	maternal plasma	60	6	49.2	14.9	769	818	*106.3*
	foetal plasma	—	6	81.5	34.9	484	501	*103.4*
207	maternal plasma	60	15	36.7	8.6	1,890	1,471	*77.8*
	foetal plasma	—	15	91.0	33.2	641	733	*114.4*
	maternal plasma +carrier phosph.	—	15	145.3	73.0	459	455	*99.1*
209	maternal plasma	40	15	41.5	17.8	45.1	31.2	*69.1*
	— —	—	52	41.5	12.1	45.1	41.3	*91.5*
210	maternal plasma	40	14	20.0	10.0	153	141	*92.2*
	— —	—	30	20.0	8.8	153	155	*101.3*

centage of phosphate was adsorbed from these solutions than from serum or plasma.

In experiments with heparinized *plasma* the results were inconsistent. Only in three out of eight cases was the specific activity lower after adsorption than before, and the difference was not as great as when serum was used. However, in these cases the blood was not collected under oil to prevent carbon dioxide loss, and this might have caused a break-down of labile phosphate compounds before the start of the experiment.

The experiments Nos. 201—206 were carried out in Baltimore on guinea pigs from the colony of the Carnegie Institution of Washington, while the rest were performed in Copenhagen on animals from the State Serum Institute. If only the results in non-pregnant animals are considered, these could also be ascribed to differences between the two strains of guinea pigs with regard to plasma phosphate, but this does not quite hold true in the pregnant animals. SACKS (1931) found a significant difference in the exchange between the plasma phosphate and the phosphate of the interstitial fluid and the tissues in two strains of rats, as indicated by the disappearance from the plasma of intravenously injected radioactive phosphate. However, differences with regard to the non-adsorbable phosphate compound might not even be due to differences between various strains of animals, but simply to the fact that it is only present in the blood under certain conditions.

Foetal blood, pooled from several foetuses of a litter in each experiment, was investigated in three cases, in none of which a non-adsorbable fraction was found.

Summary.

When serum from guinea pig blood with added radioactive phosphate was shaken with precipitated barium sulphate it was found that the exogenous phosphate was adsorbed more readily than the original serum phosphate. Control experiments with Ringer or Tyrode solutions showed no changes in the specific activity after adsorption. Experiments with heparinized plasma gave inconsistent results. Foetal plasma gave no decrease in specific activity after adsorption.

The results of these experiments give added support to the belief

that the plasma may contain a labile phosphate compound, which is non-diffusible and which is not adsorbed to barium sulphate to the same extent as inorganic phosphate, but which chemically is determined as such.

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The Conversion of [1-^{14}C] Cetyl Alcohol into Palmitic Acid in the Intestinal Mucosa of the Rat.

By

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Studies on the absorption of hydrocarbons and higher aliphatic alcohols have played an important rôle in extending our knowledge of the intestinal absorption of triglycerides. For a review cf. BERGSTRÖM and BORGSTRÖM (1954).

During recent years especially hydrocarbons have been the subject of several investigations with recently developed methods, but the mechanisms involved in the intestinal absorption of the higher aliphatic alcohols have not been studied with corresponding technics.

MUNK and ROSENSTEIN (1891) were the first to study the absorption of substances closely allied to triglycerides and resembling them in physical properties. They administered cetyl palmitate to a patient with a lymph fistula and found that the chyle contained tripalmitin but were unable to find any cetyl alcohol or cetyl palmitate. Similar results were obtained with amyl oleate. FRANK (1898) administered ethyl esters of the higher fatty acids and BLOOR (1912) fed mannite esters of lauric and myristic acid to dogs with a cannulated thoracic duct. They were both unable to demonstrate any of the corresponding alcohols or esters in the lymph fat. LYMAN (1917) fed ethyl palmitate to rats but did not find appreciable amounts of ethyl esters in the fat depots. Similar results were recently obtained with more sen-

sitive methods by BORGSTRÖM (1952 a) after feeding ethyl oleate to rats with the intestinal lymphatics cannulated.

THOMAS and FLASCHENTRÄGER (1923) reported that cetyl alcohol was absorbed to some extent in dogs and CARTER and MALCOLM (1927) and CHANNON and COLLINSON (1928) found that rats were also able to absorb this substance. In addition the latter authors carried out experiments with phytol and oleyl alcohol and absorption of both of these alcohols was found to occur in rats. MANCKE (1927) after intensive feeding of cetyl acetate to different experimental animals was unable to demonstrate any cetyl alcohol in the depot fat or milk. After feeding cetyl alcohol to a patient with chyluria he was unable to recover any cetyl alcohol in the urinary fat and he therefore concluded that cetyl alcohol was rapidly metabolized by the animal.

GARDNER (1921) discovered cetyl alcohol to be a normal but minor constituent of mammalian faeces and this finding was confirmed by SCHOENHEIMER and HILGETAG (1934). The latter authors were able to isolate cetyl alcohol not only from mammalian faeces, but also from the sterile faeces of new born infants, the intestinal wall, the faeces of dogs with biliary fistulae and the contents of sterile operative intestinal cysts. From these findings they concluded that cetyl alcohol was a secretory product of the intestinal mucosa.

The intestinal absorption of cetyl alcohol was further studied by STETTEN and SCHOENHEIMER (1940) who fed deuterium labelled cetyl acetate to rats. They showed that cetyl alcohol was well absorbed and were also able to isolate deuterated fatty acids from the carcass. The absorbed cetyl alcohol had thus been extensively metabolized in the body.

In the present study we have extended the investigations of STETTEN and SCHOENHEIMER and have thus fed [1—^{14}C] cetyl alcohol to rats having a thoracic duct fistula in an attempt to determine whether cetyl alcohol is already converted into palmitic acid during intestinal absorption and whether some alcohol is absorbed unaltered via this route.

Experimental.

[1 — ^{14}C] Cetyl alcohol was prepared by reduction of [1 — ^{14}C] palmitic acid with lithium aluminium hydride in absolute ether, m. p. 47—48° (NYSTROM and BROWN 1947 a, b). The labelled cetyl alcohol had a specific activity of about 72,000 c. p. m. per mg.

Adult male rats weighing about 250 g were used in this study. The procedure used for cannulating the thoracic duct and the treatment after operation was that earlier described by BERGSTRÖM, BLOMSTRAND and BORGSTRÖM (1954). At least 18 hours after the operation, when the animals had recovered and showed a good lymph flow, they were fed 0.5 ml of a 5 per cent solution of [1—^{14}C] cetyl alcohol in olive oil by stomach tube while under light ether anaesthesia. The lymph was collected during the next 24 hours. The animals were then killed and the whole intestinal tract removed in one piece and hydrolysed together with the faeces collected during the experiment by refluxing for 2 hours with an excess of 30 per cent KOH. The mixture was diluted with water and the unsaponifiable material extracted from the alkaline solution with light petroleum. The solution was then acidified with diluted hydrochloric acid and the fatty acids extracted with light petroleum. In some experiments the faecal matter was hydrolysed alone and extracted as described above. The amount of activity absorbed was calculated by taking the difference between the amount administered and the amount recovered from the intestinal tract and faeces.

Fractionation of lymph lipids.

The lymph fat was extracted with alcohol : ether 3 : 1 and after the evaporation of this extract in vacuo the residue was extracted with light petroleum : chloroform 2 : 1. In preliminary experiments the total fat was separated into neutral fat and phospholipids on columns of silicic acid and saponified as described by BORGSTRÖM (1952 b). It was found that the major part of the activity was obtained in the neutral fat fatty acids, about 15 per cent of the total activity in the lymph was contained in the unsaponifiable portion of the neutral fat and a small part in the phospholipid fatty acids.

In order to investigate whether some free cetyl alcohol was present in the lymph lipids a chromatographic procedure for its isolation was worked out. In experiments with pure substances it was found that on a column of silicic acid prepared according to BORGSTRÖM (1952 b), cetyl alcohol could be eluted with light petroleum : benzene 8 : 2.

The total unhydrolysed lipids from an experiment (416 mg) were dissolved in 20 ml light petroleum and put on the column prepared from 10 g of silicic acid and 5 g of Hyflo Supercel. The column was eluted with successive 100 ml portions of light petroleum with increasing percentages of benzene (fig. 1). Each 100 ml portion of the effluent was evaporated to dryness, the residue weighed and the specific activity determined by the direct plating of 1 mg of the fraction on aluminum planchets (<0.2 mg./cm.) After the triglycerides had been eluted the phospholipids were eluted with methanol, saponified, and the fatty acids extracted after acidification. With this procedure it was apparently not possible to separate free cetyl alcohol from the cholesterol esters of higher fatty acids as these are also eluted with light petroleum : benzene 8 : 2 (BORGSTRÖM 1952 c). The amount of cholesterol esters in lymph is only about 1—2 per cent of the total fat according to BORGSTRÖM

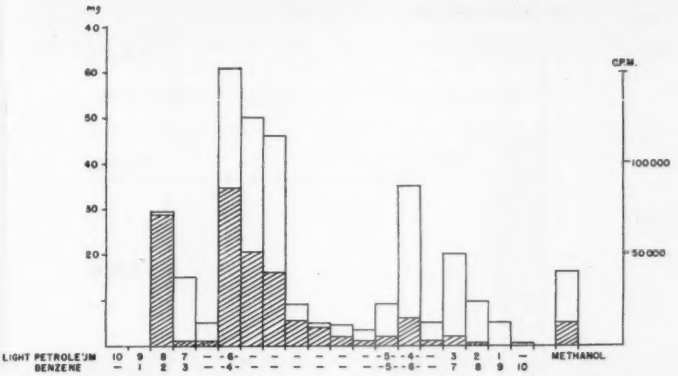

Fig. 1. Chromatogram from separation of rat thoracic lymph fat on silicic acid after feeding [1—14C] cetyl alcohol dissolved in olive oil (see text). Open columns represent the weight of each fraction and filled the total activity of each fraction.

(1952 d), but the separation method should nevertheless be able to serve as an indicator for the amount of free cetyl alcohol present even if the quantities are small. In figure 1, a fraction which represents about 15 per cent of the total activity in the lymph lipids was eluted with light petroleum : benzene 8 : 2 but the major part of the activity was localized in the triglycerides. The fractions eluted with 40 per cent and more benzene were combined and subjected to chromatography on Amberlite IRA 400 to make sure that they did not contain free fatty acids. Thereafter the neutral portion was saponified with 4 per cent KOH in absolute ethanol and the fatty acids extracted with alkaline 50 per cent ethanol. The fatty acids were then reextracted with light petroleum after acidification. The major part of the recovered activity in the lymph lipids was obtained in this fraction. Apparently the cetyl

Table 1.

Recovery of activity in lymph lipids after feeding labelled cetyl alcohol dissolved in olive oil to rats with a thoracic duct fistula.

Rat no.	Per cent of administered activity absorbed	Per cent of absorbed activity recovered in lymph lipids	Per cent of activity in lymph lipids recovered as:		
			Neutral fat fatty acids	Cetyl alcohol free	Phospho-lipid fatty acids
10.....	91.8	30.5	75.3	22.5	2.2
11.....	62.5	63.5	82.8	14.6	2.6
12.....	95.5	60.5	86.0	10.0	4.0
13.....	86.5	56.0	—	—	—

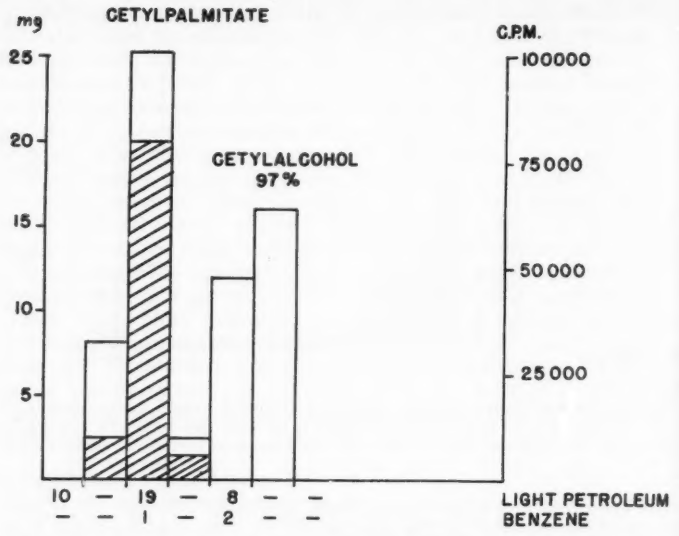

Fig. 2. Chromatogram showing separation of ^{14}C labelled cetyl palmitate from cetyl alcohol on a column of silicic acid. Open columns represent the weight of each fraction and filled the total activity of each fraction.

alcohol had been oxidized to an acid, presumably palmitic. In order to test this presumption inactive palmitic acid (50.0 mg) was mixed with neutral fat fatty acids (10.0 mg), five recrystallizations were made in aqueous acetone and the specific activity determined after every crystallization. As shown in Table 2 a the activity remained constant, indicating that the acid was palmitic acid.

In order to analyse the fraction which had been eluted with light petroleum : benzene 8 : 2 a part of this fraction (14.0 mg) was mixed with inactive cetyl alcohol (90.0 mg) and crystallized first in methanol and then five times in acetone (Table 2 b). The activity remained constant and therefore the major part of the activity in this fraction could be attributed to free cetyl alcohol. When another part of this eluate was saponified with 4 per cent KOH in absolute ethanol and the saponification mixture extracted with alkaline 50 per cent ethanol and the fatty acids reextracted after acidification with light petroleum only a very small amount of active fatty acids was obtained. The major part of the activity, however, remained in the unsaponifiable fraction. It is thus very likely that the activity in the acid portion could be attributed to fatty acids obtained from cholesterol esters or to contamination with the triglycerides.

To exclude the possibility that there was any cetyl palmitate in the lymph [C^{14}] cetyl palmitate was synthesized by refluxing [1—^{14}C]

Table 2 a.

Specific activity of neutral fat fatty acids of rat lymph after recrystallization with inactive palmitic acids.

Recrystallization No.	Weight mg	Counts/ min./mg
1.....	49.0	164
2.....	39.6	171
3.....	30.0	173
4.....	20.1	168
5.....	10.0	170

Table 2 b.

Specific activity of a fraction from chromatography of rat lymph fat eluted with light petroleum : benzene 8 : 2 after recrystallization with inactive cetyl alcohol.

Recrystallization No.	Weight mg	Counts/ min./mg
1.....	61.6	105
2.....	56.5	98
3.....	48.5	95
4.....	40.5	104
5.....	31.8	102
6.....	15.0	102

palmitic acid for 5 hours with cetyl alcohol in absolute benzene with p-toluenesulfonic acid as a catalyst (CATALINE, WORRELL, JEFFRIES and ARONSON 1944). The free acids were removed by chromatography on Amberlite IRA 400.

A mixture of inactive cetyl alcohol and labelled cetyl palmitate dissolved in light petroleum was then put on a column of silicic acid and separated by chromatography as described above. As is shown in figure 2 the whole amount of cetyl palmitate was eluted with only 5 per cent benzene. From the chromatography on total lymph fat (fig. 1) one can draw the conclusion that no cetyl palmitate had been present in the lymph.

Results.

The results of the lymph experiments are summarized in Table 1. From 63 to 96 per cent of the fed activity was absorbed. From 31 to 64 per cent of the absorbed activity was recovered in the thoracic lymph lipids. The distribution of the activity between the different fractions in the lymph lipids shows that about

80 per cent of the total activity in the lymph was found in the neutral fat fatty acids mainly as palmitic acid. About 15 per cent was present as unchanged free cetyl alcohol whereas the remainder of the activity was recovered in the phospholipid fatty acids. It must be pointed out that in the values for free cetyl alcohol in Table 1 approximately 5—10 per cent of the activity (*i. e.* about 1 % of total activity absorbed) was due to cholesterol esters of higher fatty acids (BORGSTRÖM 1952 d) and possibly a small contamination from glyceride fatty acids.

Discussion.

The absorption of 63 to 96 per cent of the fed labelled cetyl alcohol are in accordance with earlier investigations that cetyl alcohol is well absorbed in the rat (cf. CARTER and MALCOLM 1927, CHANNON and COLLINSON 1928, and STETTEN and SCHOENHEIMER 1940). These percentages obtained for [1—^{14}C] cetyl alcohol are comparable with those found after feeding labelled palmitic acid to rats (cf. BLOOM, CHAIKOFF, REINHARDT and DAUBEN 1951, BORGSTRÖM 1951, and BLOMSTRAND 1954).

In four experiments from 31 to 64 per cent of the absorbed activity was recovered in the lymph lipids. In three of these experiments a mean of 60 per cent of the absorbed activity was recovered in the lymph lipids. These values are comparable with but somewhat lower than those found by the aforementioned authors for labelled palmitic acid.

The major portion of the activity in the lymph lipids was recovered in the neutral fat fatty acids. By recrystallization with inactive palmitic acid it indicated that most of the activity in the neutral fat fatty acids could be accounted for as palmitic acid. The oxidation must thus have taken place largely during its passage through the intestinal mucosa. In the experiment showing the highest recovery of activity in the lymph about 14 mg cetyl alcohol had passed through the intestinal cells and of this amount about 12 mg had been oxidized to palmitic acid. These values give an indication of the intense metabolic activity in the intestinal cells. One cannot, however, exclude the possibility that a small part of the cetyl alcohol could already have been converted to palmitic acid in the intestinal lumen by the action of bacteria and absorbed as such. The only place in the small intestine where

there are larger amounts of bacteria, however, is in the lower portion of ileum and before the fed cetyl alcohol has reached this region the greater bulk has already been absorbed. That the oxidation of the cetyl alcohol had occurred almost exclusively in the intestinal cells is also indicated by the fact that the unsaponifiable part of the faecal matter was much more active than that of the fatty acids.

Regarding the mechanism of the oxidation of the cetyl alcohol it seems likely that palmitaldehyde is an intermediate, a hypothesis already put forth by STETTEN and SCHOENHEIMER (1940). FEULGEN, IMHÄUSER and BEHRENS (1929) and FEULGEN and BEHRENS (1938) have shown that palmitaldehyde is a normal constituent of animal tissues and in spite of the rapidity of the oxidation process it may be possible to isolate labelled palmitaldehyde from the intestinal mucosa during the absorption of [1—^{14}C] cetyl alcohol.

The finding in this investigation that free cetyl alcohol could be isolated from the chyle lipids will throw new light on the investigations by GARDNER (1921) and SCHOENHEIMER and HILGETAG (1934). These latter authors found cetyl alcohol to be a normal constituent not only of mammalian faeces but also of animal tissues. They concluded that cetyl alcohol was a normal secretory product of the intestinal mucosa. Obviously the cetyl alcohol is a normal metabolite of fat metabolism but it is very rapidly converted into other substances. From the results of STETTEN and SCHOENHEIMER (1940) it was also evident that deuterium labelled cetyl alcohol is converted into palmitic acid and even stearic acid. The reverse reaction, the conversion of labelled palmitic acid into cetyl alcohol, was also shown to take place in the intestinal tract, presumably through the action of bacteria. Preliminary results obtained in this investigation seem to indicate that a significant part of any fed cetyl alcohol reaching the large intestine is converted to palmitic acid in the large intestine by bacterial action.

From the results of this investigation it is thus clear that during the absorption of cetyl alcohol most of this compound is oxidized to palmitic acid which is subsequently incorporated into glycerides, phospholipids and cholesterol esters. A minor part is absorbed unchanged and occurs in free form in the lymph.

Summary.

1. [1—^{14}C] cetyl alcohol dissolved in olive oil was fed to rats with a thoracic duct fistula. The labelled cetyl alcohol was well absorbed.

2. More than 50 per cent of the absorbed activity was recovered from the lymph lipids. About 15 per cent of the activity was present as unchanged cetyl alcohol. The remainder had been oxidized to palmitic acid and as such incorporated into the lymph triglycerides and phospholipids in the proportions characteristic of palmitic acid.

3. From the results of this investigation it is concluded that the main part of this oxidation process takes place during the passage of the lipids through the intestinal mucosa cells.

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Sustained Potentials Evoked by Olfactory Stimulation.

By

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As shown by ADRIAN (*e. g.* 1950, 1953) periodic oscillatory potentials — induced waves — and bursts of impulses are set up within the olfactory bulb by olfactory stimuli. The present series of experiments, in which a direct coupled amplifier was used for recording the electrical activity of the bulb, has revealed that sustained potentials also occur.

The experiments were performed on rabbits under light urethane anesthesia. Odorized air or purified air was blown into the nose by a respiration pump via glass cannulas inserted into the nostrils. In a first series of experiments recording was made from the olfactory bulb with Agar-AgCl-Ag electrodes, one electrode being placed at the surface of the exposed bulb, the other at some indifferent point of the skull. When a few cc of odorized air was blown into either nostril a sustained surface-positive potential of about 0.5—1 mV was recorded from the ipsilateral bulb (see Fig. 1 A) while with pure air no response was obtained. The amplitude of this potential increased with increasing intensity of stimulation up to a given maximum. The rate of rise of the potential was found to be related to the strength of the stimulus and to the velocity of the air current. After having reached its peak the potential showed a slight decrease but then remained at a fairly constant level during the rest of the insufflation. The rate of decrease at the end of the stimulation was found to be related to the strength and duration of the

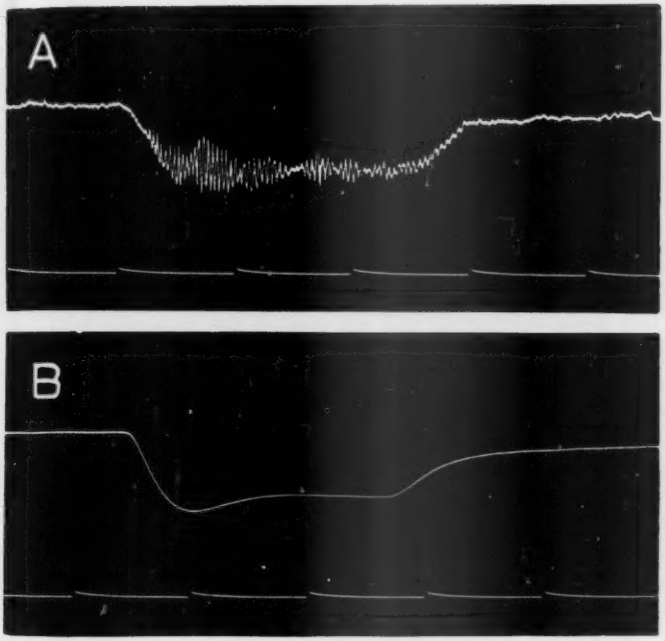

Fig. 1. Potentials recorded at olfactory stimulation. A from the olfactory bulb, B from the olfactory nerve fibres. Time marks 0.5 sec.

preceding stimulation. Sectioning of the olfactory fibres abolished the response. As typically shown in Fig. 1 A the induced waves appeared during the rising phase of the potential, reached their maximum amplitude during the plateau phase and faded out when the sustained potential decreased towards zero. They were never seen to outlast the sustained potential. In deep urethane anesthesia the potential decreased in amplitude but was still obtained after the induced waves had been blocked. At still deeper stages the sustained potential was also abolished. An abolition of the induced waves without any significant change of the sustained potential was found to occur after intravenous injection of xylocain (1—2 mg/kg).

In order to elucidate the part played by the olfactory fibres for the generation of the sustained potential monopolar recordings were also made — after removal of the bulb — from the

ends of the cut olfactory fibres at their entrance into the skull through the *foramina cribrosa*. At olfactory stimulation a slow positive potential (0.5—1 mV) was obtained (see Fig. 1 B) the shape of which was almost identical to the potential recorded from the surface of the bulb. No superimposed waves could be recorded but apart from this difference the two potentials were found to behave similarly during various experimental conditions. The characteristics of the sustained potential recorded from the olfactory fibres make it unlikely that it should represent nerve impulse activity. Furthermore, spraying of the olfactory region with a solution of cocaine of sufficient concentration (1 %) to block nerve conduction did not abolish the potential, provided the air passage through the nose was kept clear. This result taken together with earlier findings that receptor potentials are more resistant than nerve fibres to local anesthetics (KATZ 1950, GRAY and SATO 1953, OTTOSON and SVAETICHIN 1953) has led to the conclusion that the sustained potential recorded from the olfactory fibres is generated in the olfactory receptors. The experimental analysis speaks in favor of the view that the sustained potential led off from the bulb also represents the electronically or passively conducted receptor potential.

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